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Risk Assessment of Second Generation Genetically Modified Organisms

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Summary

New scientific achievements, recent advances in understanding plant metabolic pathways and biosynthesis, consumer needs and industrial demands have led to the development of second generation genetically modified plants (GM plants). In contrast to the commonly known traits - herbicide tolerance or insect resistance - GM plants of the second generation provide direct consumer benefits or are designed to simplify industrial processes and reduce production costs.

In order to get a complete picture of the status quo of second generation GM plants, different information databases were searched including global GMO authorisation registers, current datasets of field trials, and scientific literature databases. 92 worldwide authorisation entries for second generation GM plants were derived from approval GMO databases.

In many cases, there is restricted access to national databases regarding GM plant field trials. The EU and the USA databases provide accessible and evaluable datasets, and therefore these datasets were used for data collection and analysis. Stacked traits were split into single entries, which means that one trait and not one GM plant was assigned to one entry. The traits were classified in categories and sub-categories. Based on the field trial data, a trend analysis was carried out to evaluate trends for food production and for the industry.

Literature searches including Scopus, PubMed and patent databases were performed to derive information on second generation GM plants that have not yet entered the phase of field testing. Further information was derived from industry and university websites, and freely available national risk assessment documents. These data formed the basis for an analysis and discussion of second generation GM plant traits and for an evaluation of specific risk assessment requirements.

The field trial based trend analysis of the EU data conducted between 1991 and 2013 showed a maximum in 1996 and a clear downward trend beginning with 2003 for second generation GM plants. The trend analysis of the USA data was based on 5024 data entries representing field trials conducted between 1988 and 2013. This data were compared to the traits approved in the United States, which indicates correlations between field trials and approvals of GM plant traits in time periods soon after the maximum values of field trials. Furthermore higher chances for commercialisation exist for higher entry numbers in the field trial databases.

Four traits potentially providing high consumer benefits seem to be particularly interesting for future GMO approvals: enhanced vitamin content, enhanced mineral content, improved baking quality, and seedlessness of fruits. Furthermore, increased authorisation activities are expected for GM plants that modify processing characteristics or produce industrial commodities. Any predictions, however, should be treated with caution, since future developments are also influenced by factors that are difficult to account for, such as socio-economic factors.

Potential risks and negative effects for humans, potential benefits for consumers and industry, the potential use of by-products, and aspects of marketing in the European Union are described and discussed for all the different traits derived from the databases. Potential risks may arise from the oversupply of nutrients, the accidental contamination of the food/feed chain and potential unintended effects due to the genetic modification process. Potential unintended effects have to be expected for any of the second generation GM plants, and need to be evaluated on a case-by-case basis taking account of the newly introduced genetic and phenotypic characteristics of the plant such as altered metabolic pathways.

A more detailed analysis of the risk assessment was carried out for the following nine selected traits: increased oleic acid content, high amounts of stearidonic acid, very low content of coeliac-toxic epitopes (gliadin),

enhanced vitamin (provitamin content), expression of thaumatin, production of thermotolerant enzymes, high amounts of lysine, increased erucic acid content, and improved yield.

This analysis indicates a potential for improvement of current risk assessment strategies in relation to second generation GM plants. Deficits were identified regarding for example the necessity that adequate safety profiles for any compounds that are produced by GM plants need to be established, and that potentially changed metabolic profiles are addressed in the comparative assessment.

Recommendations are formulated for improvement of risk assessment strategies for second generation GM plants:

Generally, because of the high potential for unintended metabolic changes, there is a need for an appropriate nutritional-toxicological approach. A case-by-case approach should be implemented and the required toxicological testing programme be determined for each particular GM event. The occurrence of unwanted substances such as secondary plant products that do not have established safety profiles need particular attention.

It is essential to establish a valid database proving a nutrient or newly expressed protein derived from GM plants to bear no risks for causing adverse effects to development and reproduction systems and no allergological risks. Particular attention should also be paid to potential long-term adverse effects. In this respect, the safety and nutritional assessment for second generation GM plants with new substances or substantial modifications should be similar to and as strictly as for novel foods. Particularly, a risk-benefit assessment should be carried out.

A number of second generation GM plants are designed for producing commodities potentially harmful for humans or animals for non-food/feed purposes. Adequate confinement and monitoring measures need to be established preventing these substances from entering food/feed supply chains.

Further recommendations have been developed regarding the post market monitoring, exposure assessment, compositional and agronomic assessment.

Zusammenfassung

Neue wissenschaftliche Errungenschaften, jüngste Fortschritte im Verständnis von Pflanzenstoffwechselwegen und -biosynthese, Bedürfnisse von Verbrauchern und Industrie haben zur Entwicklung von gentechnisch veränderten Pflanzen (GV Pflanzen) der zweiten Generation geführt. Im Gegensatz zu den bislang bekannten Merkmalen Herbizidtoleranz und Insektenresistenz bieten GV Pflanzen der zweiten Generation direkte Vorteile für Konsumenten oder werden entwickelt, um industrielle Prozesse zu vereinfachen und Produktionskosten zu verringern.

Um ein gesamtheitliches Bild bezüglich des Status Quo von GV Pflanzen der zweiten Generation zu bekommen, wurden verschiedene Informationsdatenbanken einschließlich globale GVO Zulassungsregister, aktuelle Datensätze zu Feldversuchen und wissenschaftliche Literaturdatenbanken ausgewertet. Aus den GVO Zulassungsdatenbanken wurden 92 Einträge zu weltweiten Zulassungen von GV Pflanzen zweiter Generation erfasst.

In vielen Fällen ist der Zugriff auf nationale Datenbanken bezüglich Feldversuche von GV Pflanzen eingeschränkt. Die Datenbanken der EU und der USA bieten jedenfalls zugängliche und auswertbare Datensätze und wurden deshalb für die Datensammlung und -analyse herangezogen. Kombinierte Merkmale ("Stacked Traits") wurden in einzelne Einträge aufgeteilt, was bedeutet, dass ein Merkmal und nicht eine GV Pflanze einem Dateneintrag zugeordnet wurde. Die Merkmale wurden in Kategorien und Subkategorien klassifiziert. Basierend auf den Daten der Feldversuche wurde eine Trendanalyse durchgeführt, um Tendenzen für die Lebensmittelproduktion und die Industrie abzuschätzen.

Literatursuchen unter Einschluss von Scopus, PubMed und Patentdatenbanken wurden durchgeführt, um Informationen zu jenen GV Pflanzen zweiter Generation zu erhalten, die noch nicht in die Phase der Feldversuche eingetreten sind. Weitere Informationen wurden aus Industrie- und Universitäts-Webseiten, sowie frei zugänglichen nationalen Risikobewertungsdokumenten bezogen. Diese Daten bildeten die Grundlage für eine Analyse und Diskussion der Merkmale der GV Pflanzen zweiter Generation und der Abklärung spezieller Anforderungen der Risikobewertung.

Die auf Feldversuchen, die zwischen 1991 und 2013 durchgeführt wurden, basierende Trendanalyse der EU-Daten, zeigte für GV Pflanzen zweiter Generation ein Maximum im Jahr 1996 und einen klaren Abwärtstrend beginnend mit 2003. Die Trendanalyse der USA-Daten basierte auf 5024 Dateneinträgen zu Feldversuchen zwischen 1988 und 2013. Diese Daten wurden mit den GVO-Zulassungen in den USA verglichen, wobei sich Zusammenhänge zwischen Feldversuchen und Zulassungen von Merkmalen in Zeiträumen kurz nach den Maximalwerten der Feldversuche ableiten ließen. Weiters ergeben sich höhere Chancen auf eine Markteinführung, je größer die Zahl an Einträgen in den Feldversuchsdatenbanken ist.

Vier Merkmale, welche potentiell hohe Vorteile für Konsumenten erbringen, erscheinen besonders interessant für zukünftige GVO Zulassungen zu sein: erhöhter Vitamingehalt, erhöhter Mineralstoffgehalt, verbesserte Backeigenschaften und Kernlosigkeit von Früchten. Außerdem sind verstärkte Zulassungsaktivitäten für GV Pflanzen zu erwarten, die Verarbeitungseigenschaften modifizieren oder industrielle Rohstoffe erzeugen. Alle Prognosen sollten jedenfalls mit Vorsicht betrachtet werden, weil zukünftige Entwicklungen auch durch Faktoren beeinflusst werden, welche schwer abzuschätzen sind, wie zum Beispiel sozio-ökonomische Faktoren.

Für alle in den Datenbanken erfassten, unterschiedlichen Merkmale wurden potentielle Risiken für und negative Effekte auf den Menschen, potentielle Vorteile für Konsumenten und die Industrie, eine potentielle

Verwendung von Nebenerzeugnissen, sowie Aspekte der Vermarktung innerhalb der Europäischen Union beschrieben und diskutiert. Potentielle Risiken können von einer Nährstoffüberversorgung herrühren, einer versehentlichen Kontaminierung der Lebensmittel- oder Futtermittelkette und von etwaigen unbeabsichtigten Effekten, die auf dem Prozess der genetischen Veränderung beruhen. Derartige Effekte sind für jede GV Pflanze zweiter Generation zu erwarten und müssen unter Berücksichtigung der neu in eine Pflanze eingebrachten genetischen und phänotypischen Ausprägungen, wie zum Beispiel modifizierte Stoffwechselwege, von Fall zu Fall evaluiert werden.

Für die folgenden neun ausgewählten Merkmale wurde eine detailliertere Analyse der Risikobewertung durchgeführt: erhöhter Ölsäuregehalt, hoher Gehalt an Stearidonsäure, sehr niedriger Gehalt an mit Zöliakie einhergehenden toxischen Epitopen (Gliadin), erhöhter Vitamin- (Provitamin -) Gehalt, Expression von Thaumatin, Produktion thermotoleranter Enzyme, hoher Gehalt an Lysin, erhöhter Erukasäuregehalt und Ertragssteigerung.

Diese Analyse lässt ein Potenzial zur Verbesserung bestehender Risikobewertungsstrategien bezüglich GV Pflanzen zweiter Generation erkennen. Defizite wurden zum Beispiel identifiziert hinsichtlich der Notwendigkeit der Erstellung angemessener Sicherheitsprofile für alle Stoffe, die von einer GV Pflanze produziert werden. Und potentiell geänderte Stoffwechselprofile müssen in den vergleichenden Bewertungen ("Comparative Assessment") ausreichend Berücksichtigung finden.

Es wurden Handlungsempfehlungen zur Verbesserung von Risikobewertungsstrategien für GV Pflanzen zweiter Generation erarbeitet:

Generell besteht auf Grund des hohen Potenzials für unbeabsichtigte Stoffwechselveränderungen die Notwendigkeit eines angemessenen ernährungs-toxikologischen Ansatzes. Ein case-by-case Ansatz sollte durchgeführt und das für jeden einzelnen GVO Event notwendige toxikologische Testprogramm festgelegt werden. Besondere Beachtung erfordert das Auftreten von unerwünschten Substanzen wie sekundären Pflanzenprodukten, die keine fundierten Sicherheitsprofile aufweisen.

Es ist wichtig eine valide Datenbasis zu schaffen, die beweist, dass ein von einer GV Pflanze stammender Nährstoff oder ein neu exprimiertes Protein keine Risiken betreffend schädlicher Effekte auf Entwicklungs- und Reproduktionssysteme und allergologischen Risiken in sich bergen. Besondere Aufmerksamkeit sollte auch möglichen negativen Langzeit-Effekten gewidmet werden. Diesbezüglich muss die Sicherheits- und die ernährungswissenschaftliche Bewertung von GV Pflanzen zweiter Generation, die neuen Stoffen produzieren oder substantielle Veränderungen aufweisen, in ähnlicher Weise und ebenso streng wie für Novel Foods durchgeführt werden. Weiters sollte eine Risiko-Nutzen-Analyse vorgenommen werden.

Einige GV Pflanzen zweiter Generation werden für die Produktion von Rohstoffen für andere als Lebensmittel- oder Futtermittelzwecke konzipiert. Diese können auch potentiell schädlich für Mensch und Tier sein. Adäquate Beschränkungs- und Monitoring-Maßnahmen müssen eingerichtet werden um zu verhindern, dass diese Substanzen in die Lebensmittel- oder Futtermittelversorgungskette gelangen.

Zusätzliche Handlungsempfehlungen wurden betreffend Post Market Monitoring, Expositionsabschätzung, sowie Inhaltstoff- und agronomische Bewertung ausgearbeitet.

1 Introduction

Genetically modified plants (GM plants) of the first generation possess properties which are relevant only for agricultural praxis. Particularly, herbicide- and insecticide tolerant GM plants belong to this category, and many of these have already been authorised worldwide for food and feed, import or cultivation. GM plants of the second generation are different from first generation GM plants, because the traits are intended to provide benefits for consumers or industrial applications.

In order to gain an overview of the status quo of second generation GM plants, information was derived from online GMO databases and research of literature. The collected data were categorised, compared and used for a discussion of potential trends and future developments of GM plant traits that belong to the second generation. A discussion of the traits in relation to a consumer or industrial benefit, the potential use of waste products and the relevance for the EU market is provided.

The differences and new features of second generation GM plants make it necessary to consider changes in risk assessment strategies. The main part of this report, thus, concerns the evaluation of risks and negative effects for human health with respect to second generation GM plant traits.

In a first step, a general discussion is given on potential risks associated with these traits. In particular, the occurrence of unintended effects, the oversupply of nutrients, and the unintended commingling of food/feed supply chains is addressed.

In a second step, the risk assessment process of nine selected traits is highlighted and analysed in detail. Improvements or amendments of current GMO risk assessment strategies are discussed and specific challenges and problems targeted.

2 Status quo of second generation GM plants

2.1 Data research

To obtain all the relevant information concerning the status quo of second generation GM plants, data research was performed with regard to approvals, field trials, and the research and development status of second generation GM plants. Research for scientific literature and in the databases focussed on getting information from most reliable sources and on performing data inquires using an optimal methodology. The aim was to produce highly significant results.

Information on the current authorisation status of GM plants with second generation traits was obtained from publicly available databases (e.g. Biosafety Clearing House 2014; CERA 2014; ISAAA 2014a).

Data research of field trials was carried out using the information provided from national authorities, the European Commission's Joint Research Centre and the United States Department of Agriculture (JRC 2014; USDA 2014). These databases were already used by other research teams for studying of second generation GMO traits (Lheureux et al. 2003; ADAS 2013).

To complete data research for novel biomolecular approaches concerning development and marketing of second generation GM plant traits, literature searches were performed using online databases for scientific literature, PubMed and Scopus. Additional information was derived from the European and the United States patent databases (European Patent Office, The United States Patent and Trademark Office (USPTO)).

The broad documentation of information derived from authorisation databases, from field trial datasets, and from literature and patent research provides an excellent overview of the status quo of second generation traits in the authorisation phase as well as in the test and research and development phase.

Trait and trait names
The wording "trait" means the intended genotypic and phenotypic change in the characteristic of a plant that is introduced by a genetic modification process. The names that are used in the different databases for characterisation of the traits are called "trait names".

Correction of trait names and merging under common terms
Different trait names are used in different databases for equal traits of GM plants (e.g. amylopectin content increased vs. altered starch metabolism), and also the databases are highly inhomogeneous with respect to the naming of the traits. Therefore, correction of the trait names of the database results was sometimes necessary. However, in order to derive from the databases as much information as possible, corrections were done in such a way that keeping of detail information of the trait names had priority to the merging under common terms.

For a considerable number of tested traits, it is not possible to make direct assignments to common terms without losing relevant information. Thus, in most cases, original trait names were kept, even if an assignment could have been made to simplify data evaluation and trend analysis.

As an example, the following differences in the field trial databases are illustrated:

- JRC database: "alteration of carbohydrate composition", "amylopectin content increased"
- USDA database: "altered carbohydrate metabolism", "altered starch metabolism"

The problem with the assignment to common terms is that "altered carbohydrate metabolism" (a trait name referring to manipulation of a metabolic pathway) need not be equal to "alteration of carbohydrate composition" (a trait name referring to the changed composition in the kernel). Also, "altered starch metabolism" need not be equal to the trait name "amylopectin content increased" used in the JRC database, but could mean e.g. that starch content is increased. Therefore, the original trait name providing crucial information about a single field test was kept in most of the cases.

Handling of data entries of stacked traits

The handling of data of GM plants with stacked traits has a decisive influence on the outcome of the data research. For this reason, it was decided to treat each trait individually meaning that one trait was assigned to one entry in the database:

- GM stacked plants with second generation traits were treated as two different database entries.
- Three stacked traits were treated as three different entries,
- Four stacked traits were treated as four different entries, and so on.

Hence, the 777 entries in the EU field trial database represent 777 tested traits, with the number of EU field trials being only 473. The 5024 entries in the USA field trial database represent 5024 tested traits, with the actual number of USA field trials being only 3777.

2.1.1 Approvals

Information on the worldwide approvals of second generation GM plants was collected using the internet databases of the Center for Environmental Risk Assessment of the ILSI Research Foundation in Washington (CERA 2014) and of the International Service For The Acquisition Of Agri-Biotech Applications (ISAAA 2014a). These databases were chosen because they provide comprehensive information, are regularly updated, and can also be easily converted into MS Excel datasets suitable for further data processing and editing.

The datasets derived from CERA and ISAAA were verified and compared with the information found in the following database systems:

- Biosafety Clearing House (Biosafety Clearing House 2014)
- Risk Assessment Searching Mechanism (RASM 2014)
- GMO Compass (GMO Compass 2014)

The information derived from these approval GMO databases resulted in 92 worldwide authorisation entries for second generation GM plants, with the first approval granted in 1992 in the United States. (For results, see Chapter 2.2.1)

2.1.2 Field trials

The access to second generation GM plant field trial databases (i.e. plants that are currently in the test phase) is more limited than the access to approval databases. However, two both informative and comprehensive field trial databases are publicly available for data research: The JRC database, which presents field trial data from the EU, and the USDA database, which provides field trial data from the USA (USDA 2014).

The USA is the worldwide leading country concerning research and development of GM plants. For this reason, it can be expected, to a certain degree, that the USDA database of the Ministry of Agriculture from the United States provides highly significant data with respect to current GM plant field testing (USDA 2014).

The advantage of the USDA database lies in its integrated function and possibility to save the data as csv-files which are easily imported into Excel for sorting, searching, grouping, categorising, etc.

The database contains information of field trials in the United States for the period 1988-2013. The dataset of 25 years of data collection offers an excellent overview of the field trial development in the last decades and was mainly used to perform the trend analyses (Chapter 6).

Another important field trial database is the GM plant database of the EU, the JRC GMO Register (JRC 2014). The EU database offers field trial information for the period 1991-2013.

Field trial data of both datasets (EU, USA) were extracted, imported into Excel and stacked traits were separated to single entries, as abovementioned. Traits from the databases were categorised and built the starting point for the analysis and discussion of the new traits of second generation GMOs (Chapter 3.2). For this discussion, data from the USDA and JRC database and literature research was complemented with information from national and international websites (like ministries, universities, scientific news and patent databases). (For results, see Chapter 2.2.2)

2.1.3 Research and development

The publication databases SciVerse Scopus and PubMed were used to conduct literature searches for obtaining detailed scientific information on second generation GM plants, especially for those that have not yet exited research and development phase. Patent databases of the USA (USPTO 2013) and Europe (EPO 2014) were used for searches as well.

In a first step, the literature search process was performed in the databases without limitations, but due to the high amount of hits defined search algorithms eventually were used for a more precise research. The limitations were that only review documents and data of the period 2009-2013 were taken into account.

The results of the literature research are presented in the Annex (Chapter 9.4).

Scopus is described as the biggest database of abstracts and publications of peer reviewed journals. Scopus offers a good opportunity to analyse various science like technic, medicine, social sciences, natural sciences and human sciences.

The research was performed in November 2013 and the keywords referred to "Title/Abstract". The period of 2009-2013 was analysed. In addition, the database was accessed regularly in order to obtain updated information (Scopus 2014).

The following search algorithms were used:

i) *(TITLE-ABS-KEY("Genetically Modified Plant") OR TITLE-ABS-KEY("Genetically Modified Plants") OR TITLE-ABS-KEY("GMO Plants") OR TITLE-ABS-KEY("GMO Plant") OR TITLE-ABS-KEY("Genetically Engineered Plants") OR TITLE-ABS-KEY("Genetically Engineered Plant") OR TITLE-ABS-KEY("Transgenic Plants") OR TITLE-ABS-KEY("Transgenic Plant") AND TITLE-ABS-KEY("fatty acid") OR TITLE-ABS-KEY("fatty acids")) AND DOCTYPE(ar OR re) AND PUBYEAR > 2008*
This search algorithm was applied for combining singular and plural forms (e.g. fatty acid, fatty acids).

ii) *(TITLE-ABS-KEY("Genetically Modified Plant") OR TITLE-ABS-KEY("Genetically Modified Plants") OR TITLE-ABS-KEY("GMO Plants") OR TITLE-ABS-KEY("GMO Plant") OR TITLE-ABS-KEY("Genetically Engineered Plants") OR TITLE-ABS-KEY("Genetically Engineered Plant") OR TITLE-ABS-KEY("Transgenic Plants") OR TITLE-ABS-KEY("Transgenic Plant") AND TITLE-ABS-KEY("altered metabolism")) AND DOCTYPE(ar OR re) AND PUBYEAR > 2008*
This search algorithm was applied for terms not having plural forms (e.g. altered metabolism).

The database PubMed was founded by the National Center for Biotechnology Information (NCBI) and offers access to peer review literature with a great range of topics like biomedicine and health, a part of life sciences, sociology, chemistry and biotechnology. It also provides links to other relevant websites and to other NCBI topics about molecular biology reference sources.

The literature search was performed in November 2013. In addition, the database was accessed regularly in order to obtain updated information (PubMed 2014).

The following search algorithms were used:

i) *(Plants, Genetically Modified[MeSH]) AND ("fatty acid"[Title/Abstract] OR "fatty acids"[Title/Abstract])*
This search algorithm was applied for combining singular and plural forms.

ii) *("Plants, Genetically Modified"[MeSH]) AND "altered metabolism"[Title/Abstract]*
This search algorithm was applied for terms not having plural forms.

The patent research in the US Patent Database (USPTO 2013) was performed in November 2013 and the keywords referred exclusively to abstracts. Patents from the years 1976-2013 were included. The following search algorithms were used:

i) *(abst/"fatty acid" OR abst/"fatty acids") AND (abst/"transgenic plants" OR abst/"transgenic plant")*
This search algorithm was applied for combining singular and plural forms.

ii) *abst/"altered metabolism" AND (abst/"transgenic plants" OR abst/"transgenic plant")*
This search algorithm was applied for terms not having plural forms.

The European Patent Office (EPO) is the authority for registration of patents and brands in Europe (EPO 2014). The patent research in the EPO database was performed in January 2014 and the keywords referred exclusively to abstracts. No restrictions with respect to the publication year were done. Search algorithms were used as follows:

i) *(abst/"fatty acid" OR abst/"fatty acids") AND (abst/"transgenic plants" OR abst/"transgenic plant")*
This search algorithm was used for combining singular and plural forms.

ii) *abst/"altered metabolism" AND (abst/"transgenic plants" OR abst/"transgenic plant")*
This search algorithm was used for terms not having plural forms.

2.2 Results

2.2.1 Authorisations of second generation GM plants worldwide

Table 1 and Table 2 present information on the global authorisation status of second generation GM plants (approvals) derived from internet databases (CERA 2014; ISAAA 2014a). The data research resulted in 92 entries for authorised second generation GM plants. The tables show authorisations per year sorted by categories and sub-categories (Table 1) and authorisations per plant species sorted by categories and sub-categories (Table 2).

Table 1: Worldwide authorised second generation GM plants, sorted by year (categories and sub-categories)

Category / Sub-category*	1992	1994	1995	1996	1997	1998	1999	2000	2002	2005	2006	2007	2008	2009	2010	2011	2012	2013	Sum
agriculture															**1**	**2**			**3**
stress tolerance															1	2			3
anti-nutrient							**5**							**1**					**6**
phytate							5							1					6
nutrient/ingredient					**1**		**1**	**3**	**1**	**1**	**4**	**6**	**2**	**2**	**5**	**11**	**4**	**3**	**44**
amino acid										1	4	5	1						11
anti-allergen												1							1
fatty acid					1		1	3					1	2	5	11	4	3	31
nicotine									1										1
processing												**1**	**5**		**3**	**2**		**2**	**13**
protein altered												1	5		3	2		2	13
commodity		**2**		**2**											**1**				**5**
fatty acid		2		2															4
starch altered															1				1
storage	**1**	**5**	**3**	**8**	**1**	**1**	**2**												**21**
fruit ripening altered	1	5	3	8	1	1	2												21
Sum	**1**	**7**	**3**	**10**	**2**	**1**	**8**	**3**	**1**	**1**	**4**	**7**	**7**	**3**	**10**	**15**	**4**	**5**	**92**

* For information on the categorisation scheme for the traits see Chapter 3.

Table 2: Worldwide authorised second generation GM plants, sorted by plant species (categories and sub-categories)

Category / Sub-category*	soybean	maize	tomato	oilseed rape	melon	potato	rice	tobacco	Sum
agriculture		**3**							**3**
stress tolerance		3							3
anti-nutrient		**1**		**5**					**6**
phytate		1		5					6
nutrient/ingredient	**31**	**11**					**1**	**1**	**44**
amino acid		11							11
anti-allergen							1		1
fatty acid	31								31
nicotine								1	1
processing		**13**							**13**
protein altered		13							13
commodity				**4**		**1**			**5**
fatty acid				4					4
starch altered						1			1
storage			**19**		**2**				**21**
fruit ripening altered			19		2				21
Count	**31**	**28**	**19**	**9**	**2**	**1**	**1**	**1**	**92**

* For information on the categorisation scheme for the traits see Chapter 3.

2.2.2 Field trials of second generation GM plants (EU and USA)

2.2.2.1 EU field trials (JRC database)

Table 3 and Table 29 (see Annex, Chapter 9.1) present information on the field trial status of second generation GM plants in the EU for the period 1991-2013 based on internet database (JRC 2014). The tables list each trait that was tested in the EU taking special account of stacked traits, so that the 473 field trials resulted in 777 data entries (traits tested) (see also Chapter 2). The tables list the categories and sub-categories of traits tested per year (Table 3) and the trait names of traits tested per year (Table 29).

Table 3: Second generation GM plant traits tested in the EU, sorted by year (categories and sub-categories)

Category / Sub-category*	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	Sum
agriculture		**2**	**1**	**9**	**10**	**23**	**21**	**14**	**15**	**7**	**5**	**6**	**35**	**4**	**7**	**6**	**2**	**4**	**1**	**2**	**3**	**5**	**2**	**184**
carbohydrate altered			1	4	2	3	3	4	3		1					1								22
nitrogen utilisation						1		1				2		1								1		6
stress tolerance		1		5	4	13	11	1	2	3	3	1	5	1	4	2	1	3	1				1	62
yield increased		1			4	6	4	8	3	2	1	1	30	2	3	3		1		2	3	2	1	77
(n.a.) [+]							3		7	2		2					1					2		17
anti-nutrient			**1**	**4**	**2**	**4**	**7**	**1**	**2**	**1**	**1**							**1**			**1**	**1**	**1**	**27**
β-glucan																		1						1
gliadin																							1	1
glucosinolate									1	1														2
nitrate			1	2	2	2	1	1	1															10
nitrite				1																				1
phytate						1	5				1										1	1		9
(n.a.) [+]				1		1	1																	3
bioreactor					**2**	**1**						**2**						**2**					**1**	**8**
carbohydrate altered					1							2												3
protein altered					1	1												2					1	5
processing		**4**	**9**	**8**	**13**	**14**	**8**	**11**	**5**	**4**	**5**		**5**	**3**	**3**	**2**	**4**			**3**	**3**	**1**	**1**	**106**
amino acid								2																2
biofuel																				2				2
carbohydrate altered															1									1
fatty acid														1	2	2	2				1			8

Category / Sub-category*	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	Sum
fruit ripening altered		1	3	2	1			1																8
glutenin increased					1		1				2			1										5
lignin decreased				1	3	5	3		2	1	1		4	1			2			1		1	1	26
protein altered				1	1	1			1															4
starch altered							1																	1
starch content altered								1			1													2
yield increased						2		1																3
(n.a.) +		3	6	4	7	6	3	6	2	3	1		1								2			44
commodity	**1**	**9**	**7**	**12**	**32**	**48**	**35**	**25**	**12**	**19**	**3**	**3**	**12**	**16**	**8**	**11**	**5**	**6**	**10**	**2**	**5**	**4**		**285**
carbohydrate altered										1											1			2
fatty acid		2		2	11	10	11	2		1														39
lignin decreased				1	2	4	3		1				1	2							2	1		17
protein altered								1	2															3
starch altered	1	7	6	6	15	28	10	19	8	13	3	2	11	13	8	11	5	3	7	1	2	1		180
starch content altered						1	2	1												1		2		7
yield increased			1	3	4	5	8	2	1	3								1	2					30
(n.a.) +							1			1		1		1				2	1					7
storage		**2**	**3**	**7**	**5**	**3**	**1**	**15**	**3**	**1**	**1**													**41**
fruit ripening altered		2	3	7	5	3	1	15	3	1	1													41
nutrient/ingredient	**1**	**8**	**5**	**3**	**10**	**13**	**14**	**12**	**9**	**6**	**4**	**6**	**8**	**4**	**7**	**5**	**1**	**4**	**1**	**1**	**1**	**1**	**2**	**126**
amino acid		4	2	1	2	2	1	1	3	2			3		2	1		1	1					26
carbohydrate altered	1	3	3	2	3	5	4	9	5	4	2	2	3	3	2	1	1							53
fatty acid									1															1
oil		1			5	6	9	2			1	1	1	1		2		3		1	1			34
starch altered															1	1								2
starch content altered											1	1												2
vitamin												2	1		2							1	2	8
Sum	**2**	**25**	**26**	**43**	**74**	**106**	**86**	**78**	**46**	**38**	**19**	**17**	**60**	**27**	**25**	**24**	**12**	**17**	**12**	**8**	**13**	**12**	**7**	**777**

* For information on the categorisation scheme for the traits see Chapter 3.

\+ not available (no sub-category could be assigned to these traits)

2.2.2.2 USA field trials (USDA database)

Table 4 and Table 30 (see Annex, Chapter 9.2) present information on the field trial status of second generation GM plants in the USA for the period 1988-2013 based on internet database (USDA 2014). The tables list each trait that was tested in the United States taking special account of stacked traits, so that the 3777 field trials resulted in 5024 data entries (traits tested) (see also Chapter 2). The tables list the categories of traits tested per year (Table 4) and the trait names of traits tested per year (Table 30).

Table 4: Second generation GM plant traits tested in the USA, sorted by year (categories and sub-categories)

Category*	1988	1989	1990	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	Sum
agriculture				3	4	8	7	9	12	8	19	27	18	17	40	40	61	69	47	35	37	35	35	21	21	26	**599**
anti-nutrient										2	2	4	8	23	16	4	11	16	22	10	15	28	24	17	9	8	**219**
bioreactor							1			1										2							**4**
n.a. [+]			1		1	11	88	87	30	49	89	39	26	33	42	23	27	39	34	21	35	37	41	35	37	37	**862**
processing							4	7	2	5	26	15	17	5	4	4	7	5	13	19	18	18	10	13	19	47	**258**
commodity				3	6	17	5	12	11	3	9	15	19	49	37	61	42	50	51	30	65	105	112	98	101	110	**1011**
storage	1	2	4	6	12	25	51	37	18	25	26	21	11	6	2	4		4	7	17	5	6	6	10	5	4	**315**
nutrient/ingredient		1		3	8	28	42	47	53	42	50	56	33	54	86	85	92	172	166	116	123	130	117	90	86	76	**1756**
Sum	**1**	**3**	**5**	**15**	**31**	**89**	**198**	**199**	**126**	**135**	**221**	**177**	**132**	**187**	**227**	**221**	**240**	**355**	**340**	**250**	**298**	**359**	**345**	**284**	**278**	**308**	**5024**

* For information on the categorisation scheme for the traits see Chapter 3.

[+] not available (no category could be assigned to these traits)

3 Evaluation of second generation GM plant traits

3.1 Categorisation scheme

For a deep analysis of the characteristics of second generation GMOs it is necessary with respect to the high diversity of data to make a sound categorisation concerning the different traits. A report analysing GMO field trials in the European Union and the United States was published by the Institute for Prospective Technological Studies on behalf of the European Commission in 2003. In this report, traits were divided into two main groups: Input traits (herbicide tolerant and insect resistant traits) and output traits. Output traits were further divided into five categories: Abiotic stress/yield, modified nutrients/ingredients, industrial use, health, and other output traits (Lheureux et al. 2003). Another important GM crop information source (GMO Compass 2006) choses four categories for grouping of crop traits (except input traits): Altered composition, pharming, stress resistance, elimination of pollutants.

In our opinion, these categorisation schemes are not sufficiently informative. For instance, traits improving agronomic properties are classified in the review report's categorisation scheme as "other output traits". Furthermore, those schemes do not differentiate between the enhancement of nutrients and the reduction of anti-nutrients. Both fall into one category. Additionally, the categories "industrial use" and also "pharming" could mean traits converting plants into bioreactors or producers of commodities, but also traits that facilitate industrial processes by the production of enzymes.

For these reasons, a more refined categorisation has been developed which allows a specific allocation of individual traits and trait names, and thus improves illustration and discussion of the traits. The chosen categorisation scheme consists of seven categories, and it was avoided using a category named "other output traits". The categorised data were additionally divided into sub-categories and, if appropriate, into sub-groups.

The seven categories are titled as follows:

1) Modification of nutrients/ingredients (nutrient/ingredient)
2) Increase of storage time (storage)
3) Plants used as bioreactors (bioreactor)
4) Production of commodities for the industry (commodity)
5) Modification of processing characteristics (processing)
6) Modified agronomic properties (agriculture)
7) Elimination or reduction of anti-nutritive substances (anti-nutrient)

(The short-terms for these categories (terms in brackets) are used in the Tables.)

Description of the seven categories

"Modification of nutrients/ingredients" means GM plants providing a benefit for end consumers by alteration of levels of nutrients or ingredients. "Ingredient" is defined as any substance or product, including flavourings, food additives and food enzymes, and any constituent of a compound ingredient, used in the manufacture or preparation of a food and still present in the finished product, even if in an altered form (EC 2011). "Nutrient" means protein, carbohydrate, fat, fibre, sodium, vitamins and minerals listed in point 1 of Part A of Annex XIII to this Regulation, and substances which belong to or are components of one of those categories (EC 2011).

"Increase of storage time" means GM plants products of which have an extended shelf life. In general, this characteristic refers to fruits or vegetables with delayed ripening times and improved storage-life.

"Bioreactors" means GM plants used for production of certain substances (e.g. enzymes, active substances) which are prepared, purified and then used for industrial applications. The main use of these substances is as food/feed additives. Currently, such active substances are produced mainly by microorganisms. Examples are thaumatin or isomaltulose producing GM plants.

"Production of commodities" means GM plants that synthesise high levels of certain compounds used primarily as industrial feedstock. An example is oilseed rape producing erucic acid, which can be processed to surfactants, detergents, food and plastic additives, pharmaceuticals, etc.

"Modification of processing characteristics" means GM plants which simplify production steps. Examples are GM plants that produce thermotolerant α-amylase and simplify ethanol production.

"Modified agronomic properties" means GM plants with better growth and yield performance or qualities to withstand environmental stresses like heat, drought or salty soils.

"Elimination or reduction of anti-nutritive substance" means GM plants that have reduced levels of unwanted substances. Examples are GM potatoes with reduced alkaloid content, GM wheat without gliadine.

3.2 Description of the traits

This chapter provides an overview of the diverse traits of second generation GMOs. A sub-chapter discusses the most important issues relating to potential risks of second generation GM plants and negative effects for human health. Information on the use of by-products or waste-products of second generation GM plants is also provided in a sub-chapter.

The chapter contains descriptions of each trait, information on the global authorisation status and the EU and USA field trial status, considerations on potential benefits for consumers and the industry and deliberations on aspects of marketing in the European Union.

A discussion of the trends and future developments of traits is presented in Chapter 4, with diagrams of trait trends deduced from the field trial data and provided in Figure 6 and Figure 7.

Many of the second generation GM plant traits intend to simplify industrial processes and reduce production costs (e.g. thermotolerant amylase) which may lead to lower prices for certain food products. Also, traits with modified agronomic characteristics (e.g. increased yield), in the end, can generate benefits for consumers by lowering prices. However, these indirect consumer benefits are not particularly addressed.

3.2.1 Modification of nutrients/ingredients

3.2.1.1 GM plants with high amounts of lysine/methionine/tryptophan

Maize with expression of enhanced lysine level (event LY038) has been authorised in Canada, Australia, New Zealand, Philippines, Mexico, Colombia, Japan, United States and Taiwan. The first approval of this GMO was given in the United States in 2005. In the United States also 362 GM plants with increased amino acid level have been field tested since 1988. The crop species have mainly been maize, soybean, wheat and oilseed rape. 250 crops with different events have had increased lysine levels (e.g. soybean, maize, oilseed rape), 47 increased methionine and 65 increased tryptophan levels.

EU field trials were performed in the 1990ies with *Brassica* species synthesising lysine and methionine rich proteins. These trials were conducted in Finland, Belgium and France. In Germany, potatoes with increased methionine level were also field tested in the year 2000.

a) Description of the trait

Amino acids can be classified as nutritionally essential or non-essential for humans and animals. Essential amino acids cannot be synthesised by the body and so they have to be consumed. Some amino acids as, for example, lysine, are limited in some crops. The primary output of GM plants with high levels of lysine and methionine (and other amino acid like tryptophan and threonine) is the higher amount of these amino acids in the feeding stuff in order to optimise the nutrient supply for livestock feed (CERA 2008). Amino acids play a key role in human and animal nutrition and are important for the health maintenance (Ivanov 2013). The most prominent lysine enhanced crop GM maize event LY038 has an increased lysine level by approximately 40%.

b) Potential benefit for the industry

The main use of amino acids for the industry is as additives in animal feed, because crops such as soybean or maize are limited of the essential amino acids lysine and methionine (Ivanov 2013). GM Plants with high lysine or methionine content would improve the feed quality of poultry and pigs because in the end-product there would be no need of fortifying the feed with those essential amino acids mentioned before. Hence, the growth of the animals is optimised and the meat production is increased (Boisen 2000). This is very important for industry, because livestock production is growing rapidly as a demand for animal products. The FAO (2002) suggested that the global meat production and consumption would increase from 233 million t (2000) to 300 million t (2020). The most important amino acids for animal feed are L-lysine, L-threonine, L-tryptophan and DL-methionine (Karau 2014).

c) Potential consumer benefit

Proteins are polymers from amino acids and are important for growth, especially for toddlers, infants (as defined in EFSA (2011e)) and pregnant women. They have many different functions in the organism. For example, they act as enzymes or as energy sources, substantially for the regeneration of cells and as components of hormones (Elmadfa 2004). The hydroxyderivate of lysine is part of collagen and it is precursor of carnitine. Cysteine is built from methionine which is an essential amino acid as well (Elmadfa 2004).

Essential amino acids like methionine and lysine are important for human food because they are limited in seeds and vegetative tissues of many crop plants (Hacham et al. 2007). In the European Union and the United States, however, essential amino acids are generally provided from the diet in form of meat, eggs and milk, as well as from crop plants (Galili and Amir 2013) and there is no need for fortifying foods with amino acids. The Austrian Nutrition Report 2012 also points out that there is no problem with protein malnutrition in children, adults and seniors (Elmadfa et al. 2012). Amino acids, however, can be important in medical nutrition, particularly in parenteral nutrition. More often, amino acids are also appearing in dietary supplements for improving athletic performance (Karau 2014).

d) Relevance for the EU-market

Plants with high amounts of lysine or methionine are primarily relevant for animal feed and there may be an interest of the industry. However, an application for marketing of the GM maize event LY038 in the EU was withdrawn by the applicant before finalisation of the risk assessment.

3.2.1.2 GM plants with anti-allergenic potential

An anti-allergenic rice was approved in Japan in 2007 and permitted for cultivation as living modified organisms (LMO) for type 1 use (ISAAA 2012). Type 1 use means that, according to the Japanese Law

Concerning the Conservation and Sustainable Use of Biological Diversity, no preventive measures against dispersal of the LMOs into the environment are required (Yamanouchi 2005).

a) Description of the trait

Japanese cedar (*Cryptomeria japonica*) pollen allergy is very common in Japan and causes allergic disorders such as rhinitis and conjunctivitis (Takagi et al. 2005). In 2002, approximately 15% of the Japanese population were affected by Japanese cedar pollinosis, and in 2008 it were 26.5%. The status of patients is higher than with grass or ragweed pollinosis, which is more common in other countries (Okubo and Gotoh 2009). In Japan nearly 18% of the total land is covered by cedar forest and produce enormous amounts of pollen. Cedar pollen can cause a wide pollinosis because they can travel long distances and reach major cities, including Tokyo and Osaka (Okamoto et al. 2009).

The aim of a Japanese research team was to develop genetically modified rice containing cedar pollen peptide that can cure the Japanese cedar pollinosis by ingesting pollen antigen proteins. Because of the daily consumption of this edible vaccine, an allergic desensitisation and a lowering of the allergic symptoms could be induced. Efficacy tests were carried out in mice (Takagi et al. 2005; Takagi et al. 2006). In another study, an oral safety study in macaques, transgenic rice was steamed, mashed with distilled water and administered. The highest amount of applied rice was 6 g/kg/d. No pathological symptoms or histopathological abnormalities were observed (Domon et al. 2009).

From the present data, no information is available on the usage of this GM rice for immunotherapy in humans. It has been shown, however, that immunotherapy using allergen-specific T cell epitope peptides is an effective treatment for the control of IgE-mediated allergic diseases.

b) Potential benefit for the industry

Rice is very suitable for the production of recombinants, because it is a staple food, has a high grain yield, is easy to transform, self-pollinated, has an established production and processing system and provides a high yield of recombinant products. The advantage of cereal crops as vehicle of recombinant proteins is the direct delivery system for them, because there is no need for extraction and purification. There is also no need of cold storage because it is stable for more than a year even stored at room temperature (Takagi et al. 2006). This trait is probably solely used for medical applications, but current cultivation authorisation in Japan, however, makes this trait worth to be mentioned. If this GM rice was treated as any other rice, attention should be paid to the usage of by-products from processing steps (e.g. husk, bran, straw). For more information about waste products see Chapter 3.2.8.

c) Potential consumer benefit

Worldwide, the prevalence of allergic rhinitis and cedar pollinosis is increasing. Cedar pollinosis is a typical type 1 allergic disease (Okamoto et al. 2009). Using allergen-specific T cell epitope peptides as immunotherapy is effective for IgE-mediated allergic diseases, such as Japanese cedar pollinosis. The potential benefit lies in a lowering of the pollen induced allergic symptoms like rhinitis and conjunctivitis. Currently, allergies are treated by giving patients repeated injections with tiny amounts of pollen, or anti-histamines have to be taken from the allergy sufferer. The Japanese cedar pollen allergens, Cry j 1 and Cry j 2, are expressed with soybean seed protein glycinin in transgenic rice seed (Takagi et al. 2006; Domon et al. 2009).

d) Relevance for the EU-market

The rice producing cedar pollen peptides was developed especially for Japanese persons who are allergic against cedar. Considering that many pollen allergies also exist in Europe (grass pollen, birch pollen, conifer pollen, etc.), similar products could be of interest for the EU market. It should be noted, however, that the production of allergy vaccines and not food is considered to be the main use of GM plants expressing recombinant allergens (Marth et al. 2014).

3.2.1.3 GM plants with high oleic acid content

High oleic acid levels in seed is a characteristic of GM soybean registered in 7 countries. The first approval of high oleic acid soybean was granted in the United States in 1997. One high oleic acid GM soybean event (305423) is in the approval pipeline of the European Union and has been authorised in Australia, Canada, Mexico and the United States. This soybean 305423 from Pioneer contains the gene gm-fad2-1 fragment and the gm-hra gene introduced into the soybean genome. The gene fad2-1 is part of the coding region of the soybean ω-6 desaturase gene 1 (FAD2-1). The transcription of the gm-fad2-1 gene suppresses the transcription of the endogenous ω-6 desaturase making soybean 305423 a high oleic phenotype (Pioneer 2007).

a) Description of the trait

GM soybean MON87705 with enhanced content of oleic acid has been developed to enhance the suitability of soybean oil for food and industrial uses. The two key enzymes FATB and FAD2 which are involved in the soybean seed fatty acid biosynthetic pathway are down regulated. Hence, the monounsaturated oleic fatty acid (C18:1) is increased and the proportions of (C18:2) linoleic acid and (C16:0) palmitic acid decreased (EFSA 2012c). Field trials have already been performed in North America and Chile (Monsanto Company 2010).

Field trials with GM false flax and camelina with high oleic acid content are also known to have been conducted in the United States.

b) Potential benefit for the industry

The oil of soybean 305423 will be commercialised as high oleic soybean oil. The scope of the application is for food and feed uses (EFSA 2013b). MON87705 is planned also to be placed on the market for human consumption and animal feed. Because of the high amount of monounsaturated fatty acid and, in comparison, the decreased amount of polyunsaturated fatty acids, the oxidative stability of this soybean oil is higher. Accordingly, this oil is very suitable for bottled vegetable oil, salad dressings, mayonnaise, margarine and other food products. The use of this soybean oil for commercially frying is excluded. Due to the good lubricating properties of soybean oil it meets the criteria for the use in industrial applications without hydrogenation (EFSA 2013c).

c) Potential consumer benefit

At high temperatures, linoleic acid (as other poly unsaturated fatty acid) is transformed into trans-fatty acids which are a risk for coronary heart disease. Therefore, fewer trans-fatty acids are produced in the processing of oils because the soybeans have a significantly higher content of oleic acid and, conversely, less linoleic acid. Diets with high content of trans-monounsaturated fatty acids, like diets containing mixtures of saturated fatty acids, increases the blood total and LDL cholesterol concentration. Cohort studies show a consistent relationship between higher intakes of *trans* fatty acids and increased risk of coronary heart disease. Limiting the intake of *trans* fatty acids is a further positive effect in order to lower the plasma total cholesterol level.

d) Relevance for the EU-market

This trait is of high relevance for the European Union: EFSA evaluated and published a positive opinion for GM soybean MON87705 with high oleic acid content in 2012 (EFSA 2012c). Authorisations for soybean 305423 have already been granted in 10 countries.

3.2.1.4 GM plants with high amounts of stearidonic acid

GM with high amounts of stearidonic acid have been authorised in seven different countries (USA, Australia, Canada, Colombia, Mexico, New Zealand and South Africa). Field trial data for this specific trait are available for five entries (soybean) carried out in the year 2010.

a) Description of the trait

ω-3 fatty acids are popular because of their health benefits. Monsanto created the event MON87769, a soybean which produces stearidonic acid (SDA) (C 18:4 polyunsaturated fatty acid, ω-3 fatty acid). For the ω-3 fatty acids, eicosapentaenoic acid (EPA) and docosahexaenic acid (DHA) effects like maintenance and promotion of heart health and healthy circulation, normal cardiovascular function, eye, brain and heart health, cardiovascular health and heart health can be claimed.

b) Potential benefit for the industry

Soybean oil rich in the stearidonic acid is to improve the consumption of ω-3 fatty acids. The ω-3 fatty acid enhanced soybean oil can be incorporated into everyday food like soups, beverages, dressings, margarines, bakery products and snack bars (Monsanto Company 2014a; Monsanto Company 2014b). The soybean oil rich in stearidonic acid could be another sustainable option in comparison to the traditional one (fish) (Harris 2012).

c) Potential consumer benefit:

Since stearidonic acid is a precursor of eicosapentaenoic acid (EPA), GM soybean MON87769 should provide the consumer a higher intake of ω-3 fatty acids (Stepanek 2012). Stearidonic acid from plants is a good alternative for people who do not want consume fish like vegetarians or vegans. The Japan EPA Lipid Intervention Study (which used EPA supplementation) showed, as well as clinical trials with SDA enhanced soybean oil, that raising ω-3 fatty acid (SDA or EPA) can have clinical benefit of cardiovascular health (Harris 2012).

d) Relevance for the EU-market

Several authorisations of DHA (docosahexaenoic acid) rich plants or oils passed through the European commission as novel food ingredient in the EU. For instance, the oil rich in DHA from the microalgae *Schizochytrium sp.*, with a DHA content of 32%.

Another notification concerns Sacha inchi oil with substantial equivalence of vegetable oil derived to the seeds of flax seed oil. The Sacha inchi oil contains a high level of α-linoleic acid and a slightly higher amount of tocopherol as the flax seed oil. Another example is the novel food ingredient Echium oil, which is also rich in stearidonic acid (10% of the oil). These examples show that ω-3 fatty acids rich foods are an important issue for an optimum nutrition in Europe.

It should also be mentioned that GM soybean MON87769 with high stearidonic acid levels has been notified and is in the pipeline for authorisation in the EU indicating a high relevance for this trait.

3.2.1.5 GM plants with increased amylose content

Field trial data for this specific trait are available from the USDA database for five GM wheat plants carried out in the year 2009 to 2010. Also in the EU field trials have been performed, in Sweden in 1998, 2000 and 2004, and in the Czech Republic in 2006.

a) Description of the trait

Anti-sense technology was applied for genetically engineering of plants suppressing starch branching enzymes. This led to a reduced amylopectin accumulation in tubers and cereal grains resulting in a concomitant increase in amylose content (Carciofi et al. 2012). Such bioengineering has been successfully performed in wheat, rice, potato and barley providing increased amylose starches.

b) Potential industrial benefit

Modification of the carbohydrate metabolism like the starch or sugar composition of potatoes provides a number of industrial benefits as it can influence the taste of food, reduces the brown blotchiness or improves the browning effect on frying or roasting (GMO Compass 2010). High amylose starches provide additional health benefits and can also be used as biomaterial (Carciofi et al. 2012). The high amylose corn has also a great potential in the drug therapy because it can deliver bioactive molecules through the digestion system (Desai 2005).

c) Potential consumer benefit

A small amount of dietary starch termed "resistant starch" is not digested by humans but reaches the large intestine, where it is fermented by colonic bacteria that produce short chain fatty acids which are linked to several health benefits. Resistant starch is preferentially derived from amylose, and therefore the production of amylose-only starch granules is in the focus of interest. Potential applications are the manufacturing of novel food additives (Carciofi et al. 2012).

d) Relevance for the EU-market

Due to the relatively high number of potential benefits (food industry, biomaterial and drug therapy) this trait seems to be of relevance for the EU-market.

3.2.1.6 GM plants with fructan accumulation

Fructan accumulation in GM wheat was field tested in the USA on three occasions between the years 2008 and 2010. The EU field trial data reveal that field tests with GM sugar beet accumulating fructan were performed in the United Kingdom in 1995.

a) Description of the trait

Fructan is naturally produced by some plants like artichokes, chicory, wheat and rye. Chicory is even used commercially as fructan producing crop. However, because of the limitations seen in chicory - fructan breakdown before harvest - genetic engineering approaches for fructan production using new platform crops like sugar beet, sugarcane or rice are considered (van Arkel et al. 2013).

Sugar beet plants have been genetically modified to accumulate fructans (Pilon-Smits et al. 1999), and also GM potatoes have been developed with significant fructan expression in their tubers (Weyens et al., 2004). Fructan accumulation in GM potatoes has been associated with abiotic stress tolerance (Livingston III et al. 2009).

b) Potential industrial benefit

Fructan accumulation in plants is associated with better taste and storability, and improved nutritional value (Smeekens et al. 2000). Furthermore, enhanced fructan production will benefit the fructose sweetener industry and bring benefits for feeding stuffs (Caimi et al. 1999).

c) Potential consumer benefit

Studies demonstrate that inulin-type fructans modulate the secretion of gastrointestinal peptides which are involved in the lipid metabolism and in the regulation of the appetite. It was also shown that inulin-type fructans could reduce the risk of colon carcinogenesis (Roberfroid 2007).

d) Relevance for the EU-market

Due to the potential benefits for the food and feed industry fructan accumulation in plants could be of relevance for the EU-market.

3.2.1.7 GM plants with enhanced vitamin (pro-vitamin) content

In the United States, between 2003 to 2013, 38 field trials related to GM plants with altered vitamin levels were carried out. The vitamin alterations, more specifically, concerned carotenoid (19), tocopherol (9), ascorbate (6), and folic acid (4). The concerned plant species were e.g. cassava, maize, peanut, potato and soybean.

In the EU field trial database there are six GM plants with increased vitamin content, starting in the year 2002: Increased content of zeaxanthin in potato; increased content of lycopene in tomato and three vitamins enhanced maize (folate, β-carotene and ascorbate). In the USDA database, there are 18 entries concerning enhanced content of vitamins. The following vegetables are tested in field trials: eight potatoes, five cassavas, four maizes and one false flax. Modifications concerned the enhancement of the content of vitamin C, vitamin E, folic acid and vitamin A. The GM potatoes field tested in the EU have a modified carotenoid composition resulting in an accumulation of zeaxanthin instead of violaxanthin. More recently, a transgenic corn plant with increased levels of three vitamins has been created (JRC 2014).

Although no field test data are available for vitamin enhancement in fruits, such traits designed by means of genetic engineering have recently been developed and described for e.g. strawberry and banana. For GM strawberries a significant increase in ascorbate (vitamin C) concentration has been shown (Bulley et al. 2012). Pro-vitamin A enhancement has been studied in banana cultivars by an Australian research team (Mlalazi et al. 2012). The marketing of a β-carotene increased rice variety ("Golden Rice") in the Philippines, that has been developed to combat vitamin A deficiency in Asia, is still pending.

a) Description of the trait

Vitamins belong to the group of micronutrients which do not provide energy and are needed in relatively small amounts by organisms. They are organic substances that are essential for humans as they cannot be synthesised in sufficient quantity or not at all (Fitzpatrick et al. 2012). Vitamins have various biochemical functions and there exist many organic compounds that belong to this group. One of them are carotenoids, which are pigments that are found in many plant species, and some of which belong to the provitamin A carotenoids. These carotenoids are important for human nutrition and health.

b) Potential benefit for the industry

In Europe, the production of food supplements will be of interest, but also the bringing on the market of vitamin-enriched fruits/vegetables seems to be interesting both from a nutritional and commercial point of view.

c) Potential consumer benefit

There is no potential consumer benefit associated with vitamin enhanced GM plants for people of the European Union and the United States, as in these countries balanced diets dependency criteria are usually met.

d) Relevance for the EU-market

The intake of most vitamins is sufficient in Austria. In some cases, however, the critical intake data collected during previous reports has proven correct. These risk nutrients include vitamin D, folic acid and β-carotene. Intake of vitamin A and C is likewise critical for the elderly (Elmadfa et al. 2012). Due to the mass of fortified food, like cocoa products, cereals, milk products and food supplements, etc., the potential benefit of fruits and vegetables with high vitamin content directly consumed is limited for people in the European Union or the United States. The focus of interest in the EU rather could be to gain vitamins or provitamins to use them as food ingredients, such as carotenoids extracted from potato.

3.2.1.8 GM plants with increased mineral content

In the USA field trial database, information on field tests of the following 15 GM plants with increased mineral contents is listed: four cassava with increased iron content, one cassava with increased iron and zinc content, one cassava with increased zinc content, one lettuce with increased iron content, six maize plants with enhanced iron absorption, and two maize plants with enhanced iron bioavailability.

a) Description of the trait

Inorganic nutrients (minerals) must be taken up by plants from the environment. Genetic engineering approaches to enhance mineral nutrients are, therefore, diverse and mainly focus on strategies such as increasing the solubility of the minerals in the rhizosphere, mobilising them in the plants, transporting them to storage organs, increasing the storage capacity of the plant, and maximising bioavailability (Gómez-Galera et al. 2010). This may be achieved by introduction of genes that code for trace element-binding proteins, overexpression of storage proteins already present and increased expression of proteins that are responsible for trace element uptake into plants.

Another possibility is to introduce a protein that specifically enhances trace element absorption even in the presence of naturally occurring inhibitors, thus improving bioavailability. Other plants are genetically modified so that their contents of inhibitors of trace element absorption such as phytate are substantially reduced (please see Chapter 3.2.7.3). Increasing the expression of compounds that enhance trace element absorption such as ascorbic acid is also a possibility, although this has received limited attention so far (Lönnerdal 2003).

b) Potential benefit for the industry

A potential benefit may exist for farmers who cultivate crop lands on soils with low amounts of minerals, since these GM plants are capable of accumulating minerals and of providing good yield and quality even on low-mineral soils and of providing enhanced inorganic nutrients in feed.

c) Potential consumer benefit

Micronutrient malnutrition, especially lack of iron, zinc and vitamin A, currently afflicts more than half the world's population. To enhance the micronutrient (vitamin and mineral content) status of staple crops is considered to be one approach where crop biotechnology could generate crop varieties that could be used to strengthen food security and pre security and prevent malnutrition (FAO 2005). However, in the Western world, mineral imbalances are usually treated by dietary modifications (e.g. increasing the diversity of food intake), supplementation and commercial fortification (e.g. flour enriched with iron and folic acid, iodine enriched salt).

d) Relevance for the EU-market

The intake of most minerals is sufficient in Austria. In some cases, however, the critical intake data collected during previous reports has proven correct. These risk nutrients include calcium and iodine. Iron is also essential for children of school age and women of childbearing age. Intake of magnesium is likewise critical for male adults and older people, as well as among the elderly zinc (Elmadfa et al. 2012). Anyway, because of lack of needs, it is unlikely that mineral increased GM plants are of big interest for the EU market.

3.2.2 Increase of storage time

3.2.2.1 GM plants with reduced ethylene accumulation

Genetic engineering methods leading to reduced ethylene accumulation in plants have been applied to tomato and melon, each of which have been authorised in the United States. To some extent, ethylene reduced tomatoes have already been approved in other countries as well (Canada, Mexico, Japan and China).

EU field trials with GM plants (potato, melon, carnation) with altered ethylene biosynthesis were conducted in the years 1997, 1998 in Spain, United Kingdom and the Netherlands. The USDA database provides 225 entries for plants with altered fruit ripening and, more specifically, 31 entries for plants with altered ethylene production concerning plants like apple, tomato, potato, maize, pineapple, etc.

a) Description of the trait

Ethylene affects diverse developmental processes in plants like stress responses, senescence, and also fruit ripening, and thus inhibition of ethylene synthesis blocks ripening processes in fruits of many plant species (Klee et al. 1991). The ripening process of climacteric fruits, that continue to ripen after harvest, can be induced by ethylene dosage under controlled conditions (Isopan Insulation Pvt Ltd 2014). Non-climacteric fruits lack a climacteric rise in ethylene evolution, although some (e.g. citrus, grape) also show ripening-related symptoms in response to exogenous ethylene (Goldschmidt 1998).

Different genetic modification mechanisms have been developed to reduce ethylene accumulation in fruits, all of which aim to decrease the levels of S adenosylmethionine (SAM) in plant tissues, a precursor of ethylene, leading to extended shelf life.

b) Potential benefit for the industry

Reduced ethylene accumulation leads to improved storage characteristics as fruit ripening is delayed. This mechanism, in principle, can be introduced in any climacteric fruit and improves handling and reduces transportation losses. Possible plant species for genetic modification are, *inter alia*, apple, papaya, tomato, banana, mango, pear, passion fruit.

c) Potential consumer benefit

Consumer may benefit from the increased shelf life and extended storage conditions of the fruits.

d) Relevance for the EU-market

Slowing down the post-harvest ripening process of climacteric fruits is important as opportunities have been tried to slow down ripening after harvest in order to extent the transit and shelf life of fruits and vegetables (UK P&I Club 2006). This fact points out that GM plants with reduced ethylene accumulation could be of relevance for the European Union.

So far, however, no applications for such GM plants have been made in the EU, but field trials with plants bearing these traits were performed on European soil in the 1990ies.

3.2.2.2 GM plants with accumulation of pectin in the cell wall

GM tomatoes with accumulation of pectin in the cell wall induced by inhibition of polygalacturonase (PG) were approved in the 1990ies in the USA, Canada and Mexico. In the USA, the FLAVR SAVR^{TM} tomato (CGN-89564-2) with a reduced PG expression was the first GM plant to be commercially grown, and was approved for human consumption in 1994, in the United States (and later in Canada, Mexico and Japan). In the United Kingdom (UK), between 1996 and 1998 a GM tomato using technology similar to this tomato event was sold as concentrated tomato paste. The risk assessment was carried out by national authorities of UK prior to the introduction of Regulation (EC) No 258/97 on novel foods, and novel food ingredients (ADAS 2013). No GM plant with accumulation of pectin in the cell wall has authorisation status, at present, in the EU.

The USA field trial database has 13 entries in relation to GM plants with accumulation of pectin in the cell wall. From 1992 to 1998, in the EU similar field trials were conducted concerning GM tomato and oilseed rape.

a) Description of the trait

Pectin is a major component of the primary cell wall in dicots (plants that have two embryonic leaves) affecting cell adhesion and mechanical and textural characteristics of plant organs (Mohnen 2008). Polygalacturonases play a major role during fruit ripening of tomato. It is important for degradation of pectin in the cell wall, and thus fruit softening. In order to enhance shelf-life and to decrease transport losses, anti-sense mechanisms have been developed inhibiting polygalacturonase (PG) enzyme activity (Sheehy et al. 1988). There are other mechanism leading to accumulation of pectin in the cell wall: Suppression of pectin methylesterase is one of them, which is induced, for instance, by a specific pectin methylesterase inhibitor (SolyPMEI) known to occur in e.g. tomato and kiwi fruit (Reca et al. 2012).

b) Potential benefit for the industry

Reduced expression of polygalacturonase or inhibition by SolyPMEI leads to accumulation of pectin in the cell wall. This leads to delayed ripening of fruits and improved storage, which is a key issue in the food industry because of reduction of transportation losses and simplification of handling of fruits and vegetables (UK P&I Club 2006). Moreover, it is an important quality criterion for tomatoes to have cultivars which maintain their firmness so that they can withstand postharvest handling procedures (Kader 1986).

c) Potential consumer benefit

Consumer may also benefit from the increased shelf life and extended storage conditions.

d) Relevance for the EU-market

Since authorisation of FLAVR SAVR™ in the United Kingdom in 1996, no GM plants with altered pectin pattern traits have ever found its way into the GMO authorisation register of the EU. Since the last EU field trials date from 1998, placing on the market of GM plants with accumulation of pectin in the cell wall is not to be expected in the foreseeable future in the EU.

3.2.2.3 GM plants with reduced expression of pectate lyase

Field trials with strawberry with reduced expression of pectate lyase were carried out in the United States. In the European Union, field trials were conducted from 1995 to 1999 testing GM tomatoes and GM strawberries in Germany and Spain.

a) Description of the trait

Another principle of genetic modification for improvement of fruit firmness is reduction of pectate lyase (PL) through gene silencing. Pectate lyases act similarly to polygalacturonases degrading pectin in the cell wall. The principle of inhibiting pectate lyase has mainly been applied on strawberry fruits and leads to measurable alterations in pectin polymers in strawberry cell wall influencing the fruit ripening process (Santiago-Domenech et al. 2008). Also, ripening of tomatoes can be modified by this genetic modification approach.

Inhibition of pectate lyase is associated with improving resistance against soft-rot diseases caused by both bacteria and fungi as well. Such genetic engineering approach is described by an US patent, namely that a mutant gene coding for pectate lyase induces the loss, or partial loss, of activity of pectate lyase homologs in the plant and produces improved disease resistance (Vogel and Somerville 2005).

b) Potential benefit for the industry

Industry has an interest in altering the texture and structure of processed fruits and vegetables. The focus often lies on pectin changes, which play a role in process-induced textural changes (Van Buggenhout et al. 2009). Delayed fruit softening induced by suppression of pectate lyase gene expression, therefore, can be an advantageous trait in the processing and trading of strawberries or tomatoes.

c) Potential consumer benefit

Consumer may benefit from extended storage conditions of strawberries and tomatoes and other plants with reduced expression of pectate lyase.

d) Relevance for the EU-market

The advantages in processing, handling and transportation of fruits (e.g. strawberry) implicates the importance of the suppression of pectate lyase by genetic engineering in plants also for the EU market. However, no field trials have been conducted since 1999 in the EU and no GM plants are in the authorisation pipeline.

3.2.3 Plants used as bioreactors

3.2.3.1 GM plants with expression of thaumatin

Thaumatins are sweet proteins traditionally produced via aqueous extraction from *Thaumatococcus daniellii* Benth, a perennial tropical plant. Recombinant thaumatins are predominantly produced in yeast and barley

grains (Stoger 2012). Thaumatin production in transgenic barley and transgenic cucumber was field tested in the EU in the years 2008 and 2013, respectively (Hungary, Poland).

a) Description of the trait

Thaumatins belong to a group of extremely sweet proteins (approx. 3000 times sweeter than the same amount of sucrose) and can be produced as well from genetically modified plants. The two principal proteins found in thaumatin (thaumatin I and thaumatin II) are sequenced (Stoger 2012). Thaumatins are readily digested in the mammalian gastrointestinal tract, non-toxic, non-mutagenic and non-teratogenic in rat and mouse model systems and generally only weak sensitizers comparable to egg albumen but demonstrate no oral sensitisation in humans (Higginbotham et al. 1983).

b) Potential benefit for the industry

There is a large market for sweeteners and flavour enhancers, and therefore a cheap and economical production of thaumatins will become increasingly important for the food and feed additive sector. Recombinant thaumatins may be efficiently produced e.g. in tobacco hairy root cultures which allow the secretion of the product into the medium leading to a reduction of the costs for purification. Also a simpler reactor design (e.g. mist bioreactor) is possible. Barley grains do not contain toxic compounds per se and may be directly used as food and/or feed (Stoger 2012).

c) Potential consumer benefit

The use of biological low-calorie sweeteners may help to replace sucrose as primary sweetener, which severely compromises consumer health as one of the main causative agents for type II diabetes and obesity (Stoger 2012).

d) Relevance for the EU-market

The availability of biological low-calorie sweeteners is of utmost importance facing major health concerns due to type II diabetes and obesity (Stoger 2012). Traditionally produced (= non-GM) thaumatin is an authorised sweetener for foodstuffs and an authorised flavour enhancer in chewing gum, beverages and desserts. An application as feed additive is pending but received a positive EFSA opinion (EFSA 2011c).

3.2.3.2 GM plants with expression of glucose isomerase

Besides amylase and protease, glucose isomerase is assumed as one of the most important industrially produced enzymes today (Bhosale et al. 1996). In Sweden, a transgenic potato line containing a recombinant glucose isomerase gene was developed and field tested in 1996. The USA field trial database does not provide any information on a tested glucose isomerase GM plant trait.

a) Description of the trait

Glucose isomerase, which can be extracted from GM plants, too, catalyses the transition from D-glucose to D-fructose. D-fructose is the basic ingredient of high-fructose and enriched high fructose corn syrup, which are primarily used as substitutes for sucrose in soft drinks. Glucose isomerase, additionally, mediates the conversion of D-xylose to D-xylulose which is exploited in the production of bioethanol (Bhosale et al. 1996).

b) Potential benefit for the industry

Glucose isomerase is one of the three highest tonnage value enzymes worldwide (Circelli et al. 2010). It is primarily used for the conversion of starch to sugars. Glucose isomerase is currently produced by fermentation with several distinct bacterial species (Spök and Proksch 2012). A direct integration of the glucose isomerase gene into an appropriate starch containing plant line would facilitate fructose and ethanol production because glucose conversion would take place directly in the respective plant cell and would help to save processing and purification costs.

c) Potential consumer benefit

D-fructose plays an important role as a diabetic sweetener, as it is only slowly reabsorbed by the stomach and does not influence the glucose level in blood (Bhosale et al. 1996).

d) Relevance for the EU-market

Bioconversion of renewable biomass to fermentable sugars and ethanol is essentially important in the light of the rapid depletion of raw oil. Usable biomass consists of cellulose (40%), hemicellulose (30%) and lignin (30%) (Bhosale et al. 1996). An economic production of biofuels depends on an efficient conversion of cellulose and hemicellulose to glucose and xylulose and their subsequent fermentation to ethanol usually performed by yeasts (Bhosale et al. 1996). A combination of this multistep process using nowadays various organisms and enzymes into one plant would provide a substantial competitive advantage. Due to its widespread application as food enzyme GI from transgenic plants would be also of immanent relevance for the European food sector.

3.2.3.3 GM plants with production of isomaltulose

In Germany, a transgenic potato line synthesising high amounts of isomaltulose was field tested in 2002. The USA field trial database does not provide information on a tested isomaltulose GM plant trait.

a) Description of the trait

GM plants producing isomaltulose give a particular favourable material for use as a sucrose substitute, which is a food ingredient. Isomaltulose from non GM source has already been placed on the market as a novel food in July 2005 (EC 2005).

b) Potential benefit for the industry

Currently, industrial production of isomaltulose proceeds exclusively from sucrose with the help of immobilised microorganisms incapable of propagation. Due to the reactor-based production method, fabrication is rather costly and moreover, scale is limited. This may prevent the widespread use of isomaltulose sucrose isomers as alternative sweeteners. Transgenic plants could provide an attractive alternative to the conventional production method, since the precursor of the isomerisation (i.e. sucrose) is readily present within the plant tissue and the acreage could easily be adjusted, according to supply and demand (Bornke et al. 2002).

c) Potential consumer benefit

Sucrose is the main sweetener for use in food production. However, its high calorific value and caries inducing properties have evoked a shift in consumer preference towards alternative sweeteners (Bornke et al. 2002). The use of isomaltulose, which is a sucrose isomer, could be an alternative. A research, which was organised by a manufacturer of functional carbohydrates, was undertaken to gain a better

understanding of the emotional and psychological profiles and preferences of consumers. The results showed the attraction to isomaltulose in energy, sports and wellness drinks, but also the credibility of the next generation sugar characteristics and benefits (Food Processing 2009).

d) Relevance for the EU-market

The wide range of industrial applications as a sweetener indicates that isomaltulose expressing GM plants are of relevance for the EU-market.

3.2.3.4 GM plants with expression of xylanase

GM tobacco with an altered protein level expressing xylanase was field tested in the year 1995 in Germany. The USA field trial database does not provide information on a tested GM plant trait expressing xylanase.

a) Description of the trait

On the one hand, according to an US patent, xylanase producing traits are intended to improve the digestibility of forage plants for grass-fed animals as xylan is depolymerised by the enzyme in the plant. (Dunn-Coleman et al. 2008). On the other hand, xylanases derived from GM plants could replace microbial produced xylanases which play a significant role for several industrial applications (see below).

Research studies on xylanase producing rice and tobacco plants were carried out showing that the transgenic plants stably expressed a *xylanase B* gene of *Clostridium stercorarium*. This led to the conclusion that rice could be genetically engineered for economical production of hemicellulosic enzymes (Kimura et al. 2010). Another study investigated the expression of xylanase in poplar trees measuring an accumulation of xylanase in upper leaves with 14.4% of total amount of soluble protein. Based on these results the authors suggested transgenic poplar could be suitable for large-scale production of industrial enzymes like xylanase (Kim et al. 2012).

b) Potential industrial benefit

Xylanase enzymes that depolymerise xylan (hemicelluloses found in the plant cell wall) are currently used in the management of waste to degrade xylan to renewable fuels and chemicals, in addition to their use in food, agro-fibre, and the paper and pulp industries, where the enzymes help to reduce the environmental impact (Kim et al. 2012). In the food industry, oligosaccarides produced by xylanases are used as food additives or alternative sweeteners with beneficial properties. The sweetener xylitol (also named xylit) is produced via the monosaccharide xylose, which results from xylan depolymerisation catalysed by xylanase. Xylose can afterwards be reduced to the sugar alcohol xylitol (Motta 2013).

c) Potential user benefit

Xylitol is a natural sweetener, which occurs in many fruits and vegetables. It is used as food additive in the EU and classified as sweetener under the classification code E967. Xylitol was tested for preventing caries in adults at elevated risk of developing caries. It was found that the caries reduction was not statistically significant and that there was no indication of a dose-response effect (Bader et al. 2013).

Xylose can be used for spices and pet feed industry and in the food industry for butter and bread colouring and the preparation of fragrances. It is, moreover, used for pharmaceutical commodities and pharmaceutical intermediates (Foodchem 2014). The US patent document (Dunn-Coleman et al. 2008), however, indicates the main use of GM plants with high expression of xylanase is as forage for livestock animals.

d) Relevance for the EU-Market

Due to the variation in the relative proportions of cellulose, hemicellulose, pectin and lignin among different species of plants, the development of corresponding optimised enzyme cocktails are essential for biofuels production from lignocellulose. The potential applications of xylanases in the food industry and the bioconversion of lignocellulosic material and agro-wastes into fermentative products and the increased digestibility of animal feedstock (Motta 2013) indicates that this trait is of relevance also for the EU market.

3.2.4 Production of commodities for the industry

3.2.4.1 GM plants with increased erucic acid content

The US field trial data reveal one entry for a GM oilseed rape plant with altered erucic acid in the year 2002, but specific information on the genetic engineering method (transgene) is missing. The European JRC field trial database contains three entries for oilseed rape with increased erucic acid content. These European field trials were carried out in 1996 and 1997 in Germany and the United Kingdom.

a) Description of the trait

Erucic acid, a C 22:1 fatty acid, can be found in the seed oil of *Brassica* species like rape and crambe. In a transgenic oilseed rape an enhancement of erucic acid levels up to a maximum concentration of 72% (in total fat) was achieved by a German research team, whereas non-transgenic rape cultivars contain only about 50% erucic acid in total fat. The authors concluded further increases in erucic acid content may be expected by reducing the contents of the remaining fatty acids (oleic acid, polyunsaturated fatty acids and eicosenoic acid) (Nath et al. 2009).

b) Potential benefit for the industry

Erucic acid and its derivatives are important renewable commodities used in different industrial sectors as surfactants, detergents, plastic additives, cosmetics, pharmaceuticals, lubricants, etc. A substantial increase of the erucic acid content of the rapeseed oil would significantly reduce processing costs and could increase market prospects (Nath et al. 2009).

c) Potential consumer benefit

There are no consumer benefits associated with this trait, on the contrary, due to potential negative effects on human health, the European Union adopted a Regulation ruling that the level of erucic acid (in total level of fatty acids in the fat component) may not be greater than 5% (EEC 1976).

d) Relevance for the EU-market

The wide range of applications points to a high relevance of this trait, and taking into consideration of an expected reduction in production costs high erucic acid oilseed rape lines could be highly interesting for the EU market. However, erucic acid producing plants used for technical applications have to be separated from agricultural systems for food and feed production (see Chapter 3.3.3).

3.2.4.2 GM plants with high medium-chain saturated fatty acids (lauric and myristic acid)

There are two GM oilseed rapes with high lauric acid (C 12:0) and myristic acid (C 14:0) contents approved both in the United States and Canada with the authorisations granted in the period from 1994 to 1996. In the

United States, 246 tests with plants with altered fatty acid (oil) content have been conducted in the last 25 years, but from the available information it cannot be concluded how many test actually concerned alterations of saturated fatty acids (e.g. lauric and myristic acid). In the European Union, 25 field tests were carried out concerning GM plants with increased laurate content, all of which were oilseed rapes. These tests were carried out in the years from 1992 to 2000 in Germany, France and the United Kingdom.

a) Description of the trait

These GM oilseed rapes carry a gene coding for a thioesterase, which is an enzyme active in the fatty acid biosynthetic pathway of the developing seed. The seed contains lauric acid and, to a lesser extent, myristic acid. The processed kernels contain a similar level of lauric acid to that of coconut and palm kernel oil, which is about 45% (Calgene Inc. 1994; CERA 2011).

b) Potential benefit for the industry

The increased levels of lauric acid in the oil allow its use as a replacement for other lauric acid containing oils such as coconut and palm kernel oil in products such as confectionery coatings and fillings, margarines, spreads, shortenings and commercial frying oils (CERA 2011). It is also mentioned that lauric oil is used in the soap and detergent industries, since it has good cleansing and sudsing properties needed for shampoos, soaps, and detergents (Sayler 1997). High laurate oils also can be used for producing non-dairy coffee whitener and whipped toppings (Calgene Inc. 1994).

c) Potential consumer benefit

GM plants with high medium-chain saturated fatty acid content can be used for various industrial applications, as mentioned above. No direct consumer benefit can be deduced from the simple substitution of conventional sources of lauric acid (e.g. palm kernels) with transgenic plants.

d) Relevance for the EU-market

Due to the broad range of commercial applications as soaps and detergents, and also in the food industry, the replacement of currently used sources of lauric oil (coconut and palm kernel oil) with transgenic plants producing high medium-chain saturated fatty acids is a relevant issue for the EU.

3.2.4.3 GM plants with increased starch content

No authorisations for GM plants with increased starch content currently exist. The USA field trial database has 122 entries (traits tested) for GM plants with altered starch content. Of these 122 entries, 55 GM plants are indicated to have increased starch levels and five to have decreased levels. Plant species with increased starch content concern maize, potato, tomato, rice, and wheat. Decreased starch content concern maize, soybean and pea. For the European Union, the JRC database shows that five GM potatoes and two GM maize events with increased starch content were tested between 2005 and 2011 in Spain, Germany and Sweden.

a) Description of the trait

Starch is an important commodity for a number of industrial applications. It is especially important for the food industry. A high number of maize starch is converted to sirups or sugar. There are also several applications for starch and modified starch as food additives (Wurzburg 1972). The production of starch in plants has been stimulated by different genetic engineering approaches: The starch biosynthesis from triose-phosphate in transgenic potato tubers expressing plastidic fructose-1,6-bisphosphatase. This allows a new route of starch biosynthesis from triose-phosphates imported from the cytosol (Thorbjornsen et al.

2002). Another approach is the over-expression of sucrose synthase, which was applied to GM potatoes showing an increased starch content in tubers of about 35-55% under field condition (Baroja-Fernandez et al. 2009).

b) Potential benefit for the industry

In terms of productivity per land surface unit, transgenic potatoes with increased starch content were found to produce between 6.1 and 8.2 tonnes of starch/hectare, which is an increase in yield of about 40-85% in relation to conventional varieties. Such GM potatoes, therefore, could be of great agronomic interest and also allow reducing field area and producing agro energy (Baroja-Fernandez et al. 2009).

c) Potential consumer benefit

No consumer benefit is associated with GM plants with increased starch levels.

d) Relevance for the EU-market

Due to the advantage to produce more tonnes starch per hectare as mentioned above, it is very likely that GM plants with increased starch content are interesting to be authorised for commercial uses in the European Union.

3.2.4.4 GM plants with reduced lignin content

In the United States, 142 field tests with plants with altered lignin levels have been conducted. The field trials were carried out between 1998 and 2013 concerning the following plants: Eucalyptus (14), pine (10), switchgrass (8), poplar (6), maize (1), wheat (1). In the EU, lignin decreased traits have been field tested from 1994 to 2012 in four different countries (Spain, France, United Kingdom, Belgium). Several plant species were included: alfalfa, hybrid aspen, maize, poplar, and flax. No authorisations of GM plants with altered lignin content are known.

a) Description of the trait

Cell walls of higher plants are mainly composed of cellulose, hemicellulose and lignin. Cellulose and hemicellulose are polysaccharides with obvious value whereas lignin has negative effects on processes like the pulping of wood and stock breeding. Lignin removal is a technical challenge, and therefore several attempts have been made to alter the lignin biosynthesis pathway of biomass crops (Zhao et al. 2004; Harfouche et al. 2010). Developed biotechnological strategies include combinatorial modification of multiple lignin traits in plants, transcriptional control of lignin biosynthesis and the application of RNA interference (Zhao et al. 2004).

Maize

In transgenic maize (*Zea mays*), the down regulation of cinnamyl alcohol dehydrogenase (CAD) shows alterations in cell wall composition with higher levels of cellulose and arabinoxylans and a reduction in the total lignin content and of cell wall polysaccharides. *In vitro* degradability assays showed that midribs and stems are more degradable than wild-type plants. The CAD maize grown in the field produced higher amounts of dry biomass. Celluloses bioethanol assays revealed a higher level of ethanol production (Fornale et al. 2012).

Alfalfa

In alfalfa (*Medicago sativa*), the down-regulation of hydroxycinnamoyl CoA: shikimate hydroxycinnamoyl transferase (HCT) reduces lignin levels and improves forage quality, saccharification efficiency for bioethanol production, strongly increased digestibility and processing ability of lignocellulose. However, these modifications were also associated with unintended effects like dwarfing of changes in the plant growth (Gallego-Giraldo et al. 2011; Gallego-Giraldo et al. 2014).

Flax

Fibres of flax are used by the textile industry and contain mainly cellulose (about 70%), with hemicellulose, pectin and lignin. The presence of lignin means the fibres have worse elastic properties than non-lignocellulosic fibres, e.g. cotton fibres, which contain no lignin. The detected reduction in the lignin level in the CAD-deficient plants resulted in improved mechanical properties and improved extractability of the fibres (Wrobel-Kwiatkowska et al. 2007).

Poplars

GM trees down regulated for cinnamyl-CoA reductase have an increased ethanol yield and could be a strategy to improve biomass processing (Van Acker et al. 2014). The reduced levels of lignin and hemicellulose were associated with an increased proportion of cellulose and the chemical pulping of wood from transgenic lines revealed improved pulping characteristics, but growth was affected in all transgenic lines tested (Leple et al. 2007).

b) Potential benefit for the industry
There are several potential benefits for the reduction of the lignin level in plant walls, for instance: improved chemical pulping of wood, increased cellulose content, higher level of ethanol production, improved nutritional and energetic value and higher degradability of stems. Please see the discussion on the different plants above.

c) Potential consumer benefit

No specific consumer benefit is associated with GM plants with reduced lignin levels.

d) Relevance for the EU-market

Improvement of biomass for industrial applications like bioethanol production and the production of high quality commodities for textile industry indicate the high relevance of this trait for the global and the EU market.

3.2.4.5 GM plants with increased amylopectin

The USA field trial database shows tests for 102 traits with altered starch metabolism between 1992 and 2013. The plants predominantly concerned were maize (65), soybean (21), wheat (8), and also potato, barley, rice, and cassava. The European field trial database (JRC) provides information on tests of 168 GM plants with altered starch biosynthesis between 1992 and 2012. 88 of this 168 field tests are specifically characterised and relate to the downregulation of amylose or enhancement of amylopectin. 94% of these tests were conducted with GM potatoes.

a) Description of the trait

Inhibition of the granule-bound starch synthase by gene silencing techniques leads to plants with altered starch composition, meaning that amylopectin content is increased and amylose is decreased. Genetic modification for increasing amylopectin is a trait solely used in potatoes, as high amylopectin contents make potatoes valuable for the paper and chemical industry.

GM potato event EH92-527-1 (Amflora™) with increased amylopectin content was approved in the EU for cultivation and the use as food and feed in the year 2010. Two other potato events were also notified in 2011 but never been approved. The authorisation of placing on the market of event EH92-527-1 was annulled by the Court of Justice of the European Union in December 2013 in a decision that the European Commission departed from the rules of the authorisation procedures (General Court of the European Union 2013). At that time, the commercial use of Amflora™ had already been stopped (Kanter 2012) and the two other "starch potatoes" had been withdrawn from notification as well. No GM plants with increased amylopectin content have ever been notified elsewhere.

b) Potential benefit for the industry

Amylose, for most of the industrial applications, is undesirable because it makes the dissolved potato starch unstable. Many of the useful properties of starch come from amylopectin. Potatoes with increased amylopectin are advantageous for the paper industry, as it makes fillers and pulp adhere better, saving both energy and commodities. Other potential applications in the chemical industry are pastes, adhesives or production of packaging materials.

c) Potential consumer benefit

The notified "starch potatoes" were never intended to be used for the production of food. Also, a potential consumer benefit cannot be associated with these GM plants. The intention, however, was that cultivation and placing on the EU market of GM potatoes bearing such traits could increase the competitiveness of European starch industry.

d) Relevance for the EU-market

The use of amylopectin in industry and their needs for increased amylopectin composition shows the relevance of these traits for the EU market. Although the Amflora™ notification was withdrawn, the application itself shows the relevance of such traits for the EU market.

3.2.5 Improved processing characteristics

3.2.5.1 GM plants producing thermotolerant enzymes

GM maize producing a thermotolerant amylase (event 3272) was approved in 2007 in the United States, and also in 12 other countries since. This GM maize was notified in the EU in 2007 for all uses except cultivation, but it has not yet received positive opinion by EFSA because important parts of the risk assessment could not be finalised (EFSA 2013a).

The USA field trial data contain 106 entries of GM plants with altered processing qualities. It is unclear, however, how many of these tests relate to traits with thermotolerant enzymes. Two tests are described as tests with thermotolerant enzymes relating to barley. Another field trial concerned pepper with production of the endoglucanase enzyme. The EU field trial data provide information on one test with an α-amylase modified GM potato. This test was performed in Sweden in 1996. Seven field trials with GM potato events

tested the "improvement of processing quality" and were conducted in Germany and the United Kingdom from 1992 to 2000.

a) Description of the trait

The introduction of genes encoding for thermotolerant enzymes into the plant genome produces grain kernels (e.g. maize, barley) that can be used in ethanol production despite the high processing temperature and without the need of adding bacterial enzymes. Introduction of a thermotolerant α-amylase into the plant genome of grain kernels was successfully applied to maize (Richardson et al. 2002). Another genetic engineering approach is the expression of a thermotolerant endoglucanase in rice resulting in an enhanced conversion of cellulose into glucose (Oraby et al. 2007).

b) Potential benefit for the industry

Plants such as maize naturally contain amylases which are destroyed when corn is subjected to high processing temperatures for ethanol production, making it necessary to add microbially-produced amylase preparations. The use of maize event 3272, which expresses a highly thermotolerant amylase, bypasses this step. It is intended to be used in the dry-grind fuel ethanol industry. Plants expressing thermotolerant endoglucanases also can help produce cheap ethanol from cellulose (e.g. straw) which normally is of low economic value.

c) Potential consumer benefit

No potential consumer benefit can be associated with plants producing thermotolerant enzymes.

d) Relevance for the EU-market

Seeking approvals for maize expressing a thermotolerant amylase started in the year 2006 illustrating the importance of marketing of maize 3272 in the EU for the applicant. From the present perspective (risk assessment not finalised by EFSA), it is unclear if maize 3272 will be authorised for import and food and feed use in the EU as intended by the notifier.

3.2.5.2 GM plants with improved baking quality

In the United States, 15 field tests with plants with improved baking quality were conducted concerning three different plant species: maize, wheat and sorghum. The US field trials were performed from 2005 up to 2013 which indicates how up-to-date the aims for improvement of baking qualities by using genetic engineering methods are. Field testing in the European Union (Germany, Spain and the United Kingdom) of GM plants with improved baking quality confirms the importance of this trait. The tests were conducted between 1995 and 2001.

a) Description of the trait

The quality of wheat for food purposes (e.g. bread, pasta, noodles) derives from the contents and compositions of gluten proteins and grain hardness (Li et al. 2012b). Gluten contains hundreds of different protein components and plays a key role in determining the unique baking quality by conferring water absorption capacity, cohesively, viscosity and elasticity on dough (Wieser 2007). The composition of gluten proteins confers the unique viscoelastic property on dough. Rye and barley also contain gluten proteins (Catassi et al. 2007), although differences in quality (sub-units, amino acid sequences) and quantity (lower levels) exist, and so rye and barley do not have as good baking qualities as wheat has (Wieser 2001).

Gluten consists of monomeric gliadins and polymeric glutenins. Non-covalent bonds are important for the aggregation of gliadins and glutenins and implicate structure and physical properties of dough (Li et al. 2012b). Improvement of baking quality can be achieved by alteration of gluten protein composition, which can be induced by insertion of modified glutenin subunits successfully modifying dough mixing properties in durum wheat altering grain hardness (Li et al. 2012b). Another research team studied the introduction of gluten genes from wheat into rye. The results indicate a significant alteration in the polymerisation and composition of storage proteins in rye, which are said to be an important step towards improving bread-making properties of rye (Altpeter et al. 2004).

A second approach for improving the baking quality aims at enhancement of expression of a maltogenic amylase or a β-amylase. Maltogenic α-amylases hydrolyse amylose and amylopectin to maltose and are also able to hydrolyse maltotriose as well as cyclodextrin. This genetic engineering method was discovered and patented by Nielsen and Kjaerulff (2008).

b) Potential benefit for the industry

Non-covalent bonds are important for the aggregation of gliadins and glutenins and the physical properties of dough. Wheat gluten proteins predominantly determine the viscoelastic properties on dough (Li et al. 2012b). Improving the baking qualities by alteration of gluten protein composition is, therefore, highly attractive for any sector of food industry processing of grain flour and specialised in production of bread and other baked goods (e.g. cakes, pies).

The expression of maltogenic α-amylases in GM plants seem to be highly attractive, since they are widely used in the baking industry because of the ability to reduce retrogradation of starch (Nielsen and Kjaerulff 2008).

c) Potential consumer benefit

Improvement of baking quality in grain can upgrade dough properties leading to high quality food products.

d) Relevance for the EU-market

Due to the importance for improving the baking qualities of dough by changing gluten protein composition in cereals like wheat, rye and barley, this trait is of high relevance for the food industry in the EU.

3.2.6 Improved agronomic properties

3.2.6.1 GM plants with improved yield

The primary breeding goal in most crops of agronomical relevance is yield. There have been 77 field trials with improved yield in Europe from 1992 until 2013 and 110 in the United States from 1998 to 2010. A number of traits finally aim at increasing and stabilising yields, which can be reached by various changes in the plant genome. From 1997-2012 five field trials have been performed testing the altered development of plants. Those tests were carried out with potatoes and aspen in Europe (Spain, Sweden, Netherlands and Germany). Such field trials were conducted in the United States from 2003-2007 with Kentucky bluegrass, creeping bentgrass and maize.

a) Description of the trait

According to the database, the following approaches have been taken to increase yield in GM plants: alteration of plant architecture, interference with plant metabolism, flowering characteristics and fruiting behaviour, drought tolerance (discussed in detail in Chapter 3.2.6.2), and the alteration of ripening characteristics.

The most prominent example of altered plant architecture is decreased plant height (dwarness; "semi-dwarf varieties"), for which a classical approach is targeted interference with gibberellin metabolism (Sakamoto and Matsuoka 2004; Wang and Li 2006). Photosynthetic properties may also be altered by the optimisation of plant architecture, as for example optimal leaf habit has been shown to be advantageous for photosynthesis (Smith et al. 1997).

Alterations in plant development, in particular concerning timing of flowering and reproduction, are traits that may help to meet climatic challenges (Jung and Müller 2009). The desired phenotypes may range from earlier to delayed flowering, but include also non-flowering plants, e.g. in biennial crops like sugar beet. The manipulation of flowering characteristics is a popular trait in bioenergy and forage crops, as well as in trees. A plenty of possibilities to interfere with flowering exists, as flower induction is governed by a complex framework of genes (Samach and Smith 2013). Early onset of flowering may lead to significant yield increases (e.g. Kantolic et al. 2007). (Seed) yield is determined by the timing of flowering, but the optimal onset of flowering depends on the species, e.g. early flowering in cereals to have an extended corn filling phase, and delayed flowering when producing biomass. A number of genes involved in the regulation of flowering may be used to influence this trait (Jung and Müller 2009), but the authors state that currently examples are restricted to only a few key regulators when using transgenic approaches. They also suggest that altered flowering time may lead to new environmental risks. On the other hand, delayed or inhibited flowering is perceived as a method for gene containment, in particular in perennial and outcrossing crops.

Conferring tolerance of plants to diverse pests and environmental stress factors like salinity, pH, high and low temperature, drought, and adverse weather is another approach that contributes to crop yield (Uzogara 2000).

Traits involved in autumn phenology in trees is central to net ecosystem productivity (NEP), and represents organic C available for storage within the system or loss from it, resulting from gross primary production and total ecosystem respiration (Lovett et al. 2006). The expected effect is on biomass production and thus high yields.

Finally, alteration of photosynthetic properties may be achieved by the optimisation of plant architecture but also by increasing the photosynthetic efficiency or capacity (photosynthetic machinery, carbon flux, photorespiration, photoinhibition, assimilate partitioning, and assimilate utilisation). Enhanced photosynthesis in potato, maize, tobacco, oilseed rape and aspen, is achieved by synthesis of phytochrome A and B, respectively, suppression of shade avoidance, and, generally, an improvement of photosynthetic performances.

b) Potential benefit for the industry

Reduced plant height avoids the lodging of excessively tall plants under high nitrogen fertilisation (Sakamoto and Matsuoka 2004). Altered growth of plants (e.g. shorter plants in oilseed rape, or increased plant height in bioenergy crops or trees) facilitates on-farm management or leads to significant increase of biomass production and yield. Shorter plants are less prone to lodging, and harvesting may be more

efficient, e.g. by reducing seed losses. The trait may be combined with herbicide tolerance. Altered plant morphology may also mean that directly yield relevant parameter (e.g. tiller number, number of spikes, number of seeds, kernel weight, etc.) are altered by the introduced gene (example in the database: rice). A second aspect is increased disease resistance that is conferred by altered plant morphology, for example due to altered growth or position of leaves.

c) Potential consumer benefit

No direct consumer benefit (e.g. health benefits) is expected; however, higher yield, better management and less input may lead to reduced production costs. In some crops (e.g. cereals) the use of plant growth regulators is common practice. As these chemicals are part of the standard pesticide treatment of cereals, the use of alternate methods to decrease plant size reduces the potential of plant protection product residues on the plants. The same applies to disease tolerant plants, e.g. when less fungicide applications are needed.

d) Relevance for the EU-market

As traditional breeding programs primarily aim at yield, no significant impact of transgenic approaches is expected. For most traits traditional bred plants may be used as an alternative. However, major efforts have been made for some desired traits like drought tolerance (see Chapter 3.2.6.2), exemplified by the application for authorisation to place on the market drought-tolerant maize event MON87460.

3.2.6.2 GM plants with drought tolerance

Drought tolerance is becoming more and more important due to extended periods with scarce rainfall. It is one of the traits of general, world-wide relevance. The European database shows 70 field trials (1992-2013) specifically related to drought tolerance, in plants such as barley, flax, hybrid aspen, maize, oilseed rape, pea, poplar, potato, rice, sugar beet, sunflower, tobacco, tomato, tree tobacco, and watermelon. Field trials were conducted in Belgium, the Czech Republic, Finland, France, Germany, Hungary, Italy, the Netherlands, Poland, Spain, Sweden, and the United Kingdom. Interestingly, in the United States, only 11 field trials concerning drought tolerance are listed from 2005-2008. For comparison, 50 (1991-2011) are classified "general stress tolerance". Drought tolerant plants include tomato, (dwarf) bahiagrass, maize, petunia, potato and tall fescue. Despite the low number of field trials, maize with drought tolerance/increased water use efficiency (MON87460) was approved in the United States in 2011.

a) Description of the trait

Stress tolerance can be achieved by different approaches, like earlier maturity, but also the production of cell-protecting substances by interfering in the plant metabolism (metabolic engineering). Stress tolerant plants will give higher yields as they suffer less from adverse environmental conditions.

Drought is perceived as the most significant environmental stress in agriculture worldwide (Cattivelli et al. 2008), and together with salinity stress that usually occurs concurrently is a crucial limiting factor in agricultural production (Cominelli et al. 2013). Drought tolerance in crops of agronomical relevance becomes increasingly important due to changed environmental conditions (prolonged drought periods, especially during the corn-filling phase). It is one of the most important traits in the database and has been tackled in a wide variety of crops like maize, flax, pea, potato, barley, rice, sunflower, tomato, hybrid aspen, tobacco, maize, sugar beet, watermelon, oilseed rape, and poplar. Cold tolerance, which was engineered in potato, sugar beet, tobacco and cucumber, is frequently related to drought tolerance.

Concretely, drought tolerance of plants is approached by altering diverse key factors, among which not only functional genes but also regulatory genes like protein kinases and transcription factors have been identified in recent years and with the use of advanced technologies (Umezawa et al. 2006; Cattivelli et al. 2008). They are used to up-regulate the general stress response or to reproduce specific metabolic or physiological processes.

Two groups of candidate genes for stress-tolerance may be identified based on their biological function: single function genes, e.g. involved in osmolyte accumulation, oxygen radical scavenging, or lipid biosynthesis, but also encoding molecular chaperones, ion transporters, or channels, and regulatory genes that are involved in transcriptional or post-transcriptional regulation of gene expression (Cominelli and Tonelli 2010; Cominelli et al. 2013). The latter could be transcription factors, protein kinases, protein phosphatases and proteinases. Among these, transcription factors and components of the signal transduction pathways acting downstream (e.g. in tomato, rice and wheat) are thought to be optimal targets (Cattivelli et al. 2008). One of the major constraints is the use of a suitable promoter as particularly constitutive expression of transcription factors may severely affect plant growth under normal conditions (Cominelli and Tonelli 2010). It is expected that interference with regulatory genes will be approached instead of manipulation of single genes as stress tolerance is controlled by multiple and complex pathways (Cominelli et al. 2013). The most common strategy of engineering stress tolerance so far is the manipulation of single traits (so-called "target gene approach"; Reguera et al. 2012). So far, in most cases gene over-expression was chosen to achieve abiotic stress resistance; gene down-regulation has been attempted less frequently (Cominelli and Tonelli 2010).

Earlier development of the seed embryo (example in the database: pea) leads to more stable yield. The most sensitive phase when the basis for yield is developed is usually before the drier summer periods and during the time of sufficient humidity. In wheat yield is determined during stem elongation, whereas in maize the most sensitive developmental stage seems to be around flowering, starting from meiosis (Cattivelli et al. 2008). Moreover, water deficit inhibits photosynthesis (Cattivelli et al. 2008). Stay green plants show post-flowering drought resistance and improved grain-filling capacity under terminal drought (Cattivelli et al. 2008).

Polyamines are widely involved in stress reactions in plants and are believed to play an important role in protecting plants from various environmental stresses (Gupta 2013). Stress tolerance may be achieved, for example, by the synthesis of fructan, which may act as an osmoprotectant (den Ende 2013), and the expression of arginine decarboxylase that is a key enzyme in polyamine synthesis (Alcazar et al. 2010). About 15% of all flowering plant species, among which are cereals, vegetables, ornamentals and forage grasses of economic importance, store fructans (Vijn and Smeekens 1999). These polymers, consisting of fructose units and a terminal glucose residue, have a variety of functions in bacteria and plants, and may be engineered for diverse applications (van Arkel et al. 2013). A number of attempts to increase the accumulation of different fructan molecules may be found in the database, introducing well-studied enzymes like levansucrase or fructosyltransferases, including species like potato, ryegrass, sunflower, sugar beet, tomato, chicory, oilseed rape, and maize. However, the use of bacterial levansucrases, frequently tried in the 1990s (database: sunflower, sugar beet, potato, tomato), was not successful in terms of fructan yield and led to alterations in plant growth and aberrant phenotypes (literature reviewed by van Arkel et al. 2013). The expression of plant fructosyltransferases has proved more promising. Sugar beet, sugarcane and rice are mentioned as the most promising production platform (van Arkel et al. 2013).

The interference with photosynthesis properties (phytochrome A, overexpression of photosynthetic proteins) may lead to increased stress tolerance but also directly improve yield. Another possibility to influence drought tolerance is to reduce the number of stomata and by this to reduce water losses, or to

increase stomatal closure (Hu et al. 2006), in particular during the day. Also, increased cell wall thickness serves as a potential means to reduce water losses, which, in addition may confer resistance to plant pests (Santiago et al. 2013).

b) Potential benefit for the industry

Due to altered environmental conditions there is an increased demand for drought-tolerant plants. Depending on the approach by which drought tolerance is induced "multifunctional" plants may be produced, which are tolerant to environmental stress, pests, and produce substances with dietary impact. Here, a promising example is fructan-producing plants. The elucidation of fructan biosynthesis allows for engineering fructan producing plants for industrial purposes but also as a possible way to induce water stress tolerant plants. In the plants they have been implicated to serve as osmoprotectants (drought/cold tolerance). In plants that normally do not accumulate fructans the expression of appropriate genes may lead to enhanced tolerance against water stress. Inulin-type fructans serve as renewable commodities for production of bioethanol, fructose syrup, and specific products.

c) Potential consumer benefit

Although stress tolerance per se is not considered a trait which provides consumer benefits, there are potential side effects that could be worth mentioning:

Fructans are believed to have positive health impacts (Roberfroid 2007). Fructans provide sweet or neutral taste and have a fat-like texture, and humans are not able to digest them, which makes them interesting as low-calorie food ingredients (Vijn and Smeekens 1999). Fructan-rich diets might have health promoting effects; they are fermented by bifidobacteria in the colon. Small fructans may be used as low-caloric sweeteners, whereas long-chain fructans have organoleptic properties similar to fat. Inulin-type fructans, apart from their industrial uses, have also been introduced as functional food (increase of dietary fibre content, prebiotic properties). Polyamines have been extensively discussed in the literature concerning health benefits (Kalač and Krausová 2005; Larque et al. 2007).

d) Relevance for the EU market

For the European Union, drought tolerance seems to be relevant in the long-term, and increased interest in stress tolerant plants may be expected in the future. For some plants (e.g. trees, example in the database: aspen) transgenic approaches are potentially more attractive than traditional methods due to clearly reduced breeding times. Some progress has been made concerning the stress tolerance of plants but transfer to agricultural practice is lagging behind (Cominelli et al. 2013). A number of stresses affect field-grown plants and may cause more than additive negative effects in combination.

Food security primarily depends on strategies to increase yield stability under different stress conditions and to minimise the "yield gap" between yields in optimal and stress conditions (Cattivelli et al. 2008).

Drought tolerant maize Droughtgard™ has been commercialised recently. As a consequence an application for authorisation to place on the European market drought-tolerant maize (MON 87460) has already been risk-assessed by EFSA (application EFSA-GMO-NL-2009-70). The corresponding Scientific opinion was published in 2012 (EFSA 2012b), and its authorisation for food and feed use, import and processing, is pending whereas currently no cultivation in Europe is foreseen. A comparative study showing compositional equivalence has been published (Harrigan et al. 2009).

3.2.6.3 GM plants with improvement of the efficiency of the use of plant nutrients

Efficiency of the use of plant nutrients is exemplified by nitrogen utilisation and phosphorus use and metabolism, respectively. Nitrogen use efficiency was examined in six field trials in Sweden, France, Spain and Italy (1996-2012) in barley, maize, wheat, hybrid aspen, and tomato. In the United States, 46 trials are listed concerning altered nitrogen metabolism (1995-2010), including maize, soybean, and wheat, and one trial with maize related to nitrogen use in 2010. Phosphate metabolism has been less frequently investigated: the European database lists seven trials 1995-1998 in Germany and Portugal (potato), and also seven trials in the United States (1998-1999, maize, increased phosphorus, and 2008, tomato, enhanced phosphorus uptake).

a) Description of the trait

The relevant traits include the improvement of the efficiency of ammonium assimilation/retention, nitrogen utilisation and improved nitrogen assimilation, and the alteration of phosphate metabolism.

Nitrogen use efficiency (NUE) leads to enhanced absorption of nitrogen by crops, and thus lower levels of N-fertiliser can be applied (McAllister et al. 2012). Improving nitrogen or ammonium assimilation and retention, respectively, is approached by controlling various pathways (reviewed by McAllister et al. 2012). Among these is not only primary N metabolism, but also amino acid biosynthesis, photosynthesis and carbon metabolism, and, on a more global scale, altered expression of transcription factors and other regulatory proteins. To date, several genes that potentially improve the NUE of crop plants have been identified. Species with enhanced NUE included in the database comprise wheat (ammonium assimilation/retention, nitrogen metabolism), hybrid aspen (nitrogen metabolism), maize (nitrogen assimilation), and barley (amino acid transporters). Candidate NUE genes are involved in N uptake, assimilation, amino acid biosynthesis, C/N storage and metabolism, signalling and regulation of N metabolism and translocation, remobilisation and senescence (McAllister et al. 2012). To improve NUE in crop plants an increase in asparagine synthesis is presumed to improve growth rate, to lead to enhanced N status, and to confer N limitation tolerance, in particular under nitrogen-limiting conditions (Good et al. 2004). This approach has been taken in sunflower, maize, oilseed rape and sugar beet.

Plants frequently suffer from insufficient availability of phosphorus, which is an important plant nutrient, and therefore have developed a number of adaptations to acquire phosphate (reviewed by Raghothama 1999). Thus, there are many possibilities to increase the potential of plants to acquire phosphate, including changes in plant architecture or intervention in phosphate metabolism. Although the trait has been researched in a number of crops (Gaxiola et al. 2011) only potato is mentioned in the database. Improved phosphorus use efficiency (PUE) can be achieved by improved uptake of phosphate from soil and by improved productivity (Veneklaas et al. 2012). The largest yield benefits are expected for crops growing in soils with little P content and when little P-fertiliser is applied; when conditions for crop growth are near optimal increase PUE may lead to savings in P-fertiliser. Enhanced PUE may consequently contribute to sustainable agriculture (Gaxiola et al. 2011). Increasing PUE is attempted by strategies like improving acquisition (increased P-extraction capacity), translocation and internal utilisation of P_i (Gaxiola et al. 2011). To this end, uptake capacity was enhanced by increasing P_i transport or by organic acid production; alternatively, acquisition of phosphorus was improved by engineering plants to produce phytase or increasing plant proton-translocating pyrophosphatase activity.

b) Potential benefit for the industry (producer)

Improved use of nitrogen is both environmentally relevant and beneficial for the producer, as less external input of fertilisers is necessary. Greater N-use efficiency potentially decreases the need for N-input (fertiliser) without affecting yield (Andrews et al. 2004).

Phosphate frequently is a limiting factor in agriculture (Raghothama 1999; Raghothama 2000; Franco-Zorrilla 2004). A more efficient use of the phosphate supply reduces the need for additional fertilisation (environmental and production factor) and directly influences yield. Deficiency of phosphorus may also be observed in animal diets and has therefore been subject of research activities in order to supply animals with phosphorus sufficiently, but also to increase bioavailability. Reduced bioavailability leads to environmental impacts as manure contains high amounts of phosphorus, which may lead to local environmental pollution when disposed of. At the same time, the animals suffer from phosphorus deficiency.

c) Potential consumer benefit

Potential consumer benefits are mostly related to the environmental benefits described above.

d) Relevance for the EU market

Due to a number of benefits (environmental, animal feed, more efficient plants) traits resulting from the interference with plant nutrient metabolism are of great general importance. In some cases, only transgenic approaches will lead to the desired effect. Concerning elevated PUE research is still ongoing due to the complexity of the trait (Veneklaas et al. 2012). Nevertheless, it is expected that the trait will be important in the future.

3.2.7 Elimination and reduction of anti-nutritive substances

3.2.7.1 GM plants with reduced (1,3-1,4)-β-glucan content

In the United States, 17 field tests with thermotolerant glucanase producing GM barley were carried out between 1994 and 2007. In the European Union, in 2008, one EU field trial with GM barley with a similar trait was carried out. No authorisation of GM plants with reduced (1,3-1,4)-β-glucan content has been granted so far.

a) Description of the trait

Barley contains the following non-starch polysaccharides: cellulose, arabinoxylan, and (1,3-1,4)-β-D-glucan. Presence of (1,3-1,4)-β-D-glucan in grain fed to poultry can lead to limited nutrient uptake and decreased growth rate, because of the absence of an intestinal depolymerisation enzyme. Heat-stable (1,3-1,4)-β-glucanase depolymerising cellulose was introduced into the barley genome and it was shown that these enzyme could promote nutritional improvements of barley diets (von Wettstein et al. 2000).

b) Potential benefit for the industry

GM barley with reduced (1,3-1,4)-β-D-glucan has nutritional benefits for the chicken feeding industry. β-D-glucan is identified in barley grain as an anti-nutritive factor, because of its chemical structure. Poultry cannot easily digest this ingredient. β-D-glucan binds water in the intestine which results in the formation of gels and increased viscosity of the intestinal contents. Because of increased viscosity, the availability of the nutrients in the diet is reduced (eXtension 2013). So feeding barley diets (with high β-glucan content)

to poultry leads to sticky dropping adhering to the cloaca and causes dirty eggs. To sum up, barley with reduced β-glucan improves the feeding efficiency and the availability of nutrients for the poultry, as well as clean eggs (Newman 2014).

c) Potential consumer benefit

There is no specific consumer benefit connected with the cultivation and processing of barley with reduced β-glucan content used for animal feed.

d) Relevance for the EU-market

It can be assumed that due to the demand for high quality feeding stuff for poultry this trait can be relevant also for the European market. Moreover, field tests were performed in Germany demonstrating that cultivation of barley with reduced β-glucan content may be expected in the EU at some stage.

3.2.7.2 GM plants with a very low content of coeliac-toxic epitopes

The USA field trial database reveals that from 2011 to 2013 three GM wheat lines denoted as "biologically safe wheat lines for patients with coeliac disease" were field tested. The EU field trial database show that in the European Union one GM wheat with "reduced coeliac-toxic potential" was field tested. This field trial was conducted in 2013 in Spain.

a) Description of the trait

Coeliac disease is an immune-mediated enteropathy caused by ingestion of gluten in persons that are genetically susceptible for this health disorder. The only treatment of coeliac disease is to avoid gluten-containing food. Gluten is part of the protein fraction of the cereals wheat, rye, and barley (Catassi et al. 2007), with the gluten fraction in wheat being about 80% of total protein (Wieser 2001).

Gluten consists of monomeric gliadins and polymeric glutenins. Gliadins belong to the wheat prolamins, which is the alcohol-soluble protein fraction. α-Gliadins are the major subgroup of gluten proteins. It was assumed that only the gliadin peptides are responsible for coeliac disease (Wieser 2001), but new studies have proved that also glutenin peptides are involved (Vader et al. 2002; Catassi et al. 2007).

In order to produce wheat with a low content of coeliac-toxic epitopes, the production of gliadins is inhibited by an antisense mechanism in genetically engineered wheat varieties. Different studies are available describing the successful silencing of the expression of genes encoding a subset of gliadins in wheat (Gil-Humanes et al. 2010; Altenbach and Allen 2011). Some scientists currently try to compensate the loss of α-gliadins, and thereby the loss of visco-elastic properties, by increasing other gluten proteins not having coeliac disease-related wheat gliadin T-cell epitopes (Becker et al. 2009; Becker et al. 2012).

b) Potential benefit for the industry

The production of high quality food products from wheat flour suitable for patients with coeliac-disease could be beneficial both for food industry and people who suffer from coeliac disease.

c) Potential consumer benefit

Coeliac disease (CD) is an immune-mediated disorder caused by the ingestion of protein gluten. A lifelong, gluten-free diet is required to normalise the intestinal mucosa. Gluten is found in the grains wheat, rye, and barley, and therefore, if someone suffers from coeliac disease, the daily variety of the menu will be

restricted. Through the production of gliadin free grain flour, the making of a wide range of ready-made meal will be possible (Nameth 2010).

d) Relevance for the EU-market

Gluten-free foods are too expensive at the moment (Scott-Thomas 2014). The use of GM wheat with a very low content of coeliac-toxic epitopes provides advantages both for the food industry and concerned individuals (people affected by coeliac disease). Particularly, because there is no cure of coeliac disease and the only option for the individual is a lifelong gluten-free diet.

3.2.7.3 GM plant with improved phytase activity

Field trials have been performed in 1997, 2001, 2011 and 2012 in United Kingdom, Spain, Denmark and Czech Republic concerning barley, maize and oilseed rape.

a) Description of the trait

Phytase is an enzyme which hydrolyses phytic acid which bounds minerals in plants, like phosphorous. So, minerals are released through the activity of phytase in order to assimilate them. The introduced trait means that the GM plant (mostly barley) has an increased activity of the phytase enzyme (JRC 2014). Genetically modification of phytase expression was also studied in other transgenic crops such as maize, rice and oilseed rape (Zhang et al. 2010; Ali et al. 2013; Wang et al. 2013a; Wang et al. 2013b).

b) Potential benefit for the industry

Mainly phosphorus (P) of the minerals is important for the animal feeding, because it is a part of the energy metabolism. For that reason at present phosphors or phytase are supplemented to animal feeding. The supplementation of them is not required through to the high native content of phytase activity

(Hansen 2013). Another reappraisal shows that, the impact between dietary phytate and exogenous phytase in feed are equivocal in the aspects of efficient protein digestibility in pigs and poultry. A review on released phosphorus in feed in comparison to negative amino acid digestion through improved phytase activity is required (Selle et al. 2012).

c) Potential consumer benefit

With regard to phytate in food it is mentioned that, on the one hand, improved phytase activity in grain would improve the bioavailability of phosphorus, calcium, iron, zinc as well as amino acids, but, on the other hand, phytate has also positive health effects (SFGate 2014). However, there is no obvious consumer benefit as the improved phytase activity trait mainly effects the feeding industry (Ministry of the Environment of the Czech Republic 2011).

d) Relevance for the EU-market

It is a relevant topic for the animal feeding industry, because phytase producing GM plants can eliminate or reduce the supplementation of inorganic phosphorus in feeds for monogastric animals.

3.2.7.4 GM plants with down regulation of glucosinolate

Glucosinolate reduced GM oilseed rape has been field tested in Belgium in the years 1999 and 2000. No specific information on this trait can be derived from the US field trial database.

a) Description of the trait

Brassicales are plants which have a high glucosinolate content. For plants, the glucosinolate system protects against herbivore attacks, and is implicated in host-plant recognition by specialised predators. Glucosinolates are present in all parts of the plant. The level of glucosinolates varies in different tissues at different developmental stages and can be significantly reduced by genetic engineering.

b) Potential benefit for the industry

Oilseed rapes contain glucosinolates which is a toxin for cattle, and so this crop is unsuitable for livestock feed. During processing of the plant, like producing of oil, the glucosinolate remain stable in the press cake of oilseed plants. By using the press cake as protein rich animal feeding stuff metabolites of glucosinolate as thiocyanates, isothiocyanates, nitriles, 5-vinyl-2-oxazolidinethione and 5-vinyl-1,3-oxyzolodine-2-thione are occurred. This has adverse effects through changes in thyroid function, as palatability, reduction in performance (milk and eggs), decreased growth and reproductive rate, for cause. Ruminants are less sensitive to dietary glucosinolate as pigs or poultry. Reducing glucosinolate, therefore, is a meaningful benefit for the animal feed industry. Pressing residue or the whole plant, without glucosinolate, could be used as forages.

c) Potential consumer benefit

GM plants with reduced glucosinolate levels will primarily be used for animal feeding stuffs. Thus, glucosinolates may appear in animal-based food such as milk and eggs, but the concentration is very low. Usually, the measured concentration in animal-based food products is much lower than in vegetables for human consumption. Therefore, the only potential consumer benefit may be that undesirable fishy taint in animal-derived products can be avoided (EFSA 2008).

d) Relevance for the EU-market

The relevance applies to farmers which cultivate the protein-rich rapeseed plant as forage. The oil seed plant can be a protein source for livestock (e.g. cattle), but with the glucosinolate must be fed in limited quantities (CORDIS 2012).

3.2.8 Use of waste products or by-products

Waste products or by-products of plant origin are mainly produced by industrial processes and also agriculture. Farm-wastes include straw, hay, crop residues or silage. Organic waste is produced by paper mills or the timber industry, and, of course, the food industry (e.g. by processing of vegetables and fruits). Additional waste products are created by restaurants and other places where meals are prepared and served (Cooperband 2002).

It is assumed that the same waste products are produced by GM plants and that they are used in a similar way. This is confirmed by the fact that notifiers normally tend to point out that no specific conditions are warranted or required for the placing on the market of a GM crop event (e.g. maize event) for all uses as any other crop (maize) plant (mostly with the exception of cultivation in the EU).

According to the field trial databases of the European Union, the following plant species were used most for developing second generation traits: potato (322), oilseed rape (108), tomato (54), maize (52), sugar beet (35), rice (33), tobacco (32), and hybrid aspen (32); followed by plant species with counts well below 30 such as wheat, sunflower, barley, flax, etc.

In the United States field trial database, for development of second generation traits the following plant species were used most: maize (2120), soybean (1168), tomato (484), potato (317), oilseed rape (115), alfalfa (100), tobacco (93), and wheat (93). Other plant species with counts well below 100 as apple, cassava, sorghum, barley, melon, safflower, rice, cotton, etc.

The list of waste products, or by-products is wide-ranging from, amongst others, pulp waste (e.g. potato, sugar beet, citrus), wheat or rice bran, potato or orange peels, liquid whey, tea leaves or coffee beans, to old bread (Mukherjee et al. 2008). Many of the industrial waste products provide good sources for animal feed as can be seen from the long list provided by the EU Catalogue of feed material (EC 2013b). For example, by-products of vegetable oils derived from e.g. soybean, rapeseed, sunflower are oil cakes that are, after drying, employed as animal feed. Alternatively, they can be utilised for the production of vegetarian or vegan products. Besides the use as feeding stuffs, waste of by-products may be a source of soil amendments like fertilizers or compost (Cooperband 2002).

The question is, are there specific applications aiming at the use of waste or by-products for second generation GM plants? Press cakes of GM oilseed plants expressing essential amino acids like lysine in high amounts yield high quality animal feeding stuffs, but these GM plants are designed for the use as animal feed, and therefore the oil cake does not constitute a typical by-product. GM plants with enhanced vitamin or mineral content may find its way into the feed chain as well, and so may other second generation GM plant traits.

The use as animal feed seems to be most important for waste or by-products occurring from cultivation and industrial processing of GM crops. Therefore, the nutritional impact of each specific trait on livestock needs to be adequately evaluated before bringing on the market of a second generation GM plant and derived products. According to the Canadian Food Inspection Agency, this should include, amongst others, comparative assessments, dietary exposure, toxicology and allergenicity data, livestock feeding trials, and an evaluation of environmental safety (CFIA 2014).

3.3 Potential risks and negative effects for human health

This section provides an overview on the most notable issues concerning potential risks associated with second generation GM plant traits. A comprehensive discussion in relation to the risk assessment of selected GM plant traits with a focus on negative health effects, including exposure assessment, is presented in Chapter 5.

Potential risks in relation to GM plants can evolve from intended effects, that are effects aimed at by genetic engineering approaches, and also from unintended effects. Unintended effects are effects resulting from genetic modification that are unwanted, mostly unknown and unexpected. Many parts in the risk assessment of GM plants mainly deal with the identification and evaluation of unintended effects: e.g. analysis of flanking regions, comparative and toxicity assessment (EFSA 2011a).

Second generation GM plants are considered to be produced causing modifications to endogenous metabolism and physiology of host plants. Therefore, it has been discussed more recently that the current approach of the GMO risk assessment should be broadened with the extent of further testing depending on the trait expressed (ADAS 2013).

The following issues that are considered most important with respect to safety aspects of second generation GM plants are discussed in scientific literature and should be recognised:

- Comparative safety assessment plays a key role for second generation GM plants. This was acknowledged by different research groups noting that significances in composition were expected to be observed for nutritionally enhanced crops and should be assessed on a case-by-case basis (Glenn 2008; ADAS 2013). The selection of appropriate comparators is central to the comparative approach (EFSA 2011b), and particularly the choice of an appropriate comparator is of great importance for the safety assessment of nutritionally modified GM plants (Codex Alimentarius Commission 2003; ADAS 2013).

- The comparative assessment is also seen as the most reliable method to identify unintended effects caused by the genetic modification of the plant genome. These potentially result in pleiotropic effects like changes in metabolite levels or an unwanted expression of novel fusion proteins (Filipecki and Malepszy 2006; ADAS 2013).

- The selection of appropriate comparators is central to the comparative approach (EFSA 2011b), and particularly the choice of an appropriate comparator is of great importance for the safety assessment of nutritionally modified GM plants (Codex Alimentarius Commission 2003; ADAS 2013).

- The case-by-case approach should be seen in context of the proposed use of the food product in the diet and dietary exposure (Glenn 2008).

- A particular focus should be paid to the hazard identification stage as second generation GM traits may not have appropriate comparators, and the challenge rests in accurately identifying hazards and predicting levels of exposure for such GM traits (ADAS 2013). In case that no appropriate comparator is available, according to Regulation (EU) 503/2013, "A *comparative safety assessment cannot be made and consequently a safety and nutritional assessment of the genetically modified food or feed shall be carried out as for novel foods*" (EC 2013a).

- Minute amounts of compounds contaminating a dietary supplement can negatively affect human health. Therefore, the comparative assessment should be supplemented by extensive safety testing in case of the genetic manipulation aims at modifying plant secondary metabolism (Schubert 2008).

- Foods derived from GM plants modified for nutritional or health benefits may benefit certain populations, while other populations may be at risk from the same food. Information about the known patterns of use and consumption of a food should be used to estimate the intake of the food derived from the GM plant. Nutritional implications of the altered nutrient profile can be derived from the expected intake of the food (Codex Alimentarius Commission 2003).

- More than one chemical form of the nutrient can be expressed by second generation GM plants as a result of the modification and these may not be characterised from a nutrition perspective (Codex Alimentarius Commission 2003).

- For GM plants that are produced for industrial purposes only and that are not intended to be approved for food and feed applications, accidental intake by humans, livestock and wildlife animals, exposure of farmers and workers handling the GM plants, and exposure of people living in the vicinity is a particular issue to be taken into consideration (EFSA 2009b). This only concerns second generation GM plants producing commodities used in non-food/feed industrial applications and when no waste products are used as feed.

These issues regarding potential risks associated with second generation GM plants and derived food and feed products raise different questions. For a discussion of these questions the following three key areas in connection with the marketing of second generation GM plants have been identified:

1) Unintended effects due to the genetic modification
2) Possible oversupply of certain nutrients
3) Accidental contamination of food/feed

3.3.1 Unintended effects

Unintended effects are changes in the phenotype, response or composition in a GM plant which go beyond the intended effects of the genetic modification meaning that they do not fulfil the original objectives of the genetic modification (Cellini et al. 2004; EFSA 2011a).

Current standards for GMO risk assessment are using the concept of substantial equivalence (OECD 1993) for the purpose of identifying intended and unintended differences between GM plants and their comparators (EFSA 2011a). This is also reflected in Regulation (EU) No 503/2013 and the requirement for performing a comparative analysis. Unintended effects are regarded to be consistent differences between the GM plant and a conventional counterpart which is a non-GM genotype with a genetic background as close as possible to the GM plant (EFSA 2011b). Unintended effects may be caused by the site of transgene integration, potentially resulting in changed metabolism, novel fusion proteins, or other pleiotropic effects that could compromise product safety (Szwacka et al. 2012).

Regarding comparative assessment, which is considered a key element for nutritionally enhanced second generation GM plants (Glenn 2008), there is an evident problem that second generation GM plant traits may not have an appropriate comparator. The difficulty of the implementation of comparative assessment studies on second generation GM plants lies in the fact that many of them are substantially modified regarding composition and metabolism. The currentness of this problem is illustrated by the fact that EFSA called for tender for a review report addressing issues for the risk assessment of GM plants in cases where a comparative assessment was not fully applicable (EFSA 2012a). The review report suggested that second generation GM plant traits (output traits) might induce major changes in the host plant potentially resulting in substantial modifications to the endogenous metabolism and physiology of the plant. A further suggestion was that validated, rigorous and functional methods to verify compositional safety should be developed (ADAS 2013).

Examples of substantially modified GM plants can even be found in the global authorisation database (see Chapter 2.2.1):

Soybean event MON87769 was modified to express high levels of a polyunsaturated ω-3-fatty acid (stearidonic acid) in the seed at concentrations of approximately 20-30% of total fatty acids. The genetic modification - the expression of proteins desaturating certain endogenous fatty acids - led to changes in the fatty acid patterns so that additional fatty acid analyses were included in comparative analysis. For instance, the known propensity of unsaturated acids to form trans-isomers was evaluated. Trans-stearidonic and trans-α-linolenic acid were found to be increased, although it was concluded MON87769 soybean oil would not have negative impacts for consumers as the level of trans-fatty acids would make only a minor contribution to the overall trans-fatty acid content of foods (Food Standards Australia New Zealand 2011a). It was also found that the MON87769 soybean contains higher levels of α-linolenic acid and palmitic acid, and lower levels of oleic acid and linoleic acid (Food Standards Australia New Zealand 2011b).

Another GM soybean, event 305425, showed unintended effects as well. These, amongst other, concerned changes in the levels of odd chain fatty acids (C17:0, C17:1, C19:1). The applicant argued that the chemical properties of the modified, newly introduced GM-HRA enzyme had changed because of the replacement of one amino acid (EFSA 2013c). It was also discussed that there appear to be no published studies describing the catabolism of these odd chain fatty acids in mammals (EFSA 2013c) giving indication of existent data gaps in relation to safety aspects of food products derived from second generation GM plants. The final conclusion, however, was that replacement of vegetable oils with oil derived from soybean 305423 would increase odd chain fatty acids intake but these changes would be small and without impact on health and nutrition. Compositional differences were also found for monounsaturated and ω-6 polyunsaturated fatty acids (EFSA 2013c).

These two examples show that unanticipated changes in the plant metabolism are to be expected from genetic modification processes aiming at alteration of plant metabolic pathways. Both these soybean events are currently in the EU pipeline for approval.

Potential occurrence and problems with unintended effects of nutritionally enhanced GM crops have been described by several groups of scientific researchers:

Bohme et al. (2007) studied genetically modified myristic-acid rich oilseed rape and the nutritive value tested in efficacy studies in ten pigs each over the growing finishing period revealed a glucosinolate increase from 12.4 µmol/g in the parental plant to 19 µmol/g (dry matter) in the GM plant. The consequence was that with the feeding of this GM oilseed rape feed intake and weight gain decreased presumably due to the increasing glucosinolate.

Unintended effects observed in GM cucumber and tomato expressing thaumatin II gene were also discussed. It was found that GM plants with a high content of recombinant thaumatin contained a low content of total soluble protein in leaves and fruits, and also that GM cucumber with different transgene location possessed a specific metabolic profile. Moreover, it was noted that biosynthesis and expression of a new protein was an obvious cost to a plant and could be the reason for a decrease in amino acid levels (Szwacka et al. 2012).

Another study dealing with the potential for unintended effects in the context of GM plants with novel traits was presented for a transgenic wheat expressing a high molecular weight glutenin subunit. It was mentioned that changing biochemical pathways was an effective way for enhancing food sensory quality, but it could also lead to unintended effects. It was, furthermore, remarked that unintended effects on starch structure-function needed to be thoroughly risk assessed and that detailed knowledge of unintended effects was needed to inform end-users of possible changes in the flour product. To achieve this, several comparative analyses were carried out including, amongst others, X-ray diffraction and glucan chain distribution (Beckles et al. 2012).

Unanticipated metabolic changes and pleiotropic effects due to interactions between marker genes or their regulatory elements and genetic elements at the insertion site need also to be considered in the risk assessment of second generation GM plants. For instance, the bialaphos resistance gene (bar) was found to have pleiotropic effects when transgenic *Arabidopsis* lines were examined by microarray analysis (Miki et al. 2009). According to the USA field trial data (USDA database), the *bar* gene was used as selectable marker in at least 58 US field trials between 1988 and 2013.

Unintended effects in relation to RNAi based (anti-sense) techniques can also be a notable issue for the risk assessment of GM plants. It is noteworthy that anti-sense techniques have been used for the development of a wide range of second generation GM plants, some of which were approved by national authorities for commercial uses: tomatoes with extended shelf life (e.g. FLAVR SAVR™), soybeans with high oleic acid content (e.g. 305423), a potato with increased amylopectin levels (Amflora™), or a tobacco with reduced nicotin levels.

Concerning unintended effects upon consumption of plants generated by RNAi based (anti-sense) techniques, there is conflicting evidence available and supporting experimental data are lacking (Parrott et al. 2010; Heinemann et al. 2013): RNA as part of the human and animal diet is assumed by several authors to have a history of safe consumption concerning dsRNA and derived small RNAs (e.g. siRNA, miRNA, hpRNA) (Ivashuta et al. 2009; Parrott et al. 2010). There is no experimental evidence available in support of this assumption (Heinemann et al. 2013). RNA in the mammalian gastro-intestinal tract is supposed to be readily degraded by pancreatic nucleases and it is assumed that there is only a remote chance for survival of RNA fragments retaining biological activity (Parrott et al. 2010). However, it has been shown ribonucleotides may have an effect on the mammalian immune, hepatic and gastro-intestinal systems (Carver 1994).

Possible negative effects for the environment need also be considered in the risk assessment of second generation GM plants, as it seems to be highly unclear what the consequence would be if these were to be used as chemical factories to produce compounds used as commodities, vitamins and chemicals for industrial applications. Besides avoiding of gene flow, potential negative effects on wild fauna and flora and changes on biodiversity should be in the focus of the environmental risk assessment. Any deliberate release of second generation GM plants into the environment needs to be adequately monitored, and particularly for any identified risks a case-specific monitoring plan has to be implemented (EFSA 2010a).

3.3.2 Possible oversupply of certain nutrients

The Codex Alimentarius Commission (2003) states that GM food modified for nutritional or health benefits can be useful for certain populations, but that other populations may be at risk from the same food. This is a notable issue to be considered in the risk assessment of second generation GM plants.

The following transgenic plant traits that provide health benefits are of relevance regarding potential risks of an oversupply of nutrients: GM plants with high amounts of lysine and methionine, GM plants with high amounts of ω-3 fatty acids (e.g. stearidonic acid), GM plants with increased vitamin or mineral content.

In the European Union there is no need for fortifying foods with amino acids. This holds also true for Austria where there is no problem with protein malnutrition (Elmadfa et al. 2012). However, an oversupply with amino acids seems to be a factor that should not be neglected in countries like the United States, Canada or the European Union. This is confirmed and discussed by a number of studies:

Churchward-Venne et al. 2012 (2012) mentioned stimulation effects by amino acids of muscle protein synthesis independently of hormones and of suppression of muscle protein breakdown, but they pointed out that some athletes, in the United States and Canada, tended to consume protein in amounts that were far in excess of the current recommended dietary allowance of 0.8 g per kg per day. Amino acid requirements estimates were calculated by Young and Borgonha (2000) concluding that the daily required amounts for lysine being about 30 mg per kg, and methionine being about 13 mg per kg for adults. (The amino acid requirements of children/adolescents are higher).

Another study confirmed that the highest amino acid intakes occur with free amino acid supplements consumed by athletes who believe that the amino acids will benefit them in training and/or performance. With respect to risks of toxicity, this study, furthermore, stated that with increasing intakes of an amino acid, oxidation of the amino acid would increase. If the metabolic limit to oxidise the amino acid was reached, this inflection point would identify the upper limit for the amino acid. Above the upper limit, the potential risk of adverse effects and toxicity might increase (Pencharz et al. 2008). However, an upper limit for lysine is not mentioned in this paper.

A lysine enhanced GM maize (event LY038) has been authorised for commercial uses (as feeding stuff) in several countries (United States, Canada, Australia, etc.). The risk assessment on this GM plant was published by Health Canada (2006) concluding that the lysine levels were increased by approximately 40% and there was no adverse impact expected.

Fortification of food products with vitamins in the European Union and the United States seems to be of higher importance than fortification with indispensable amino acid. This is confirmed by the mentioned risk nutrients vitamins A, C and D, and folic acid (Elmadfa et al. 2012). Because of many products are currently fortified with vitamins in the EU, oversupply is sufficiently discussed. A statement published by the German Nutrition Society pointed out that if high-dose supplements were taken and fortified foods were consumed, excessive intake levels could occur, and that these might present a health risk. It was also mentioned that for some nutrients (e.g. vitamins A and D), the gap between the recommended intake and the tolerable upper intake level was very low (Bechthold et al. 2012).

A prominent example of a GM plant with high vitamin content is "Golden Rice" producing β-carotene (maximum 30.9 µg/g dw) (Paine et al. 2005) which was developed for persons who suffer from vitamin A deficiency. With respect to a potential oversupply with β-carotene, Blomhoff (2001) noticed that animal studies had shown β-carotene was not mutagenic or teratogenic, but, based on the knowledge that adverse effects had been reported in prospective randomised studies, he concluded high doses of β-carotene of about 20-30 mg were not be recommended until the safety of such doses could be established. Blomhoff (2001), furthermore, noted that the safe dose of vitamin A depended on the physical form of the vitamin preparation.

From these statements, it becomes clear that oversupply with nutrients produced by nutritionally enhanced GM plants could be an important issue, especially in case of inherent properties of these plants (particularly the increased compound) are not systematically and comprehensively risk assessed. Any potential risks caused by consuming of excess food supplements (fortified with amino acids, vitamins, or minerals) are, however, not different between GM plant derived products and conventional food products. If no food supplements are produced by extraction, purification and addition, but the food is consumed directly (e.g. fruits and vegetables) or indirectly (e.g. processed food, refined oil), a comprehensive risk assessment needs to be carried out on a case-by-case basis taking into consideration the known patterns of use and consumption of the food for estimating the intake of the food derived from the GM plant (for groups of populations) and the chemical properties and levels of the nutrients expressed in the GM plant (Codex Alimentarius Commission 2003).

3.3.3 Accidental contamination of food/feed

Some of second generation GM plants are produced for industrial purposes only and not intended to be approved for food and feed applications. A prominent example is the GM potato with increased amylopectin content (Amflora™) which is advantageous for paper processing and was notified for food uses (threshold 0.9%), because it could not be excluded that the GM potato event and derived products of the starch processing might be used as, or be present in food (EFSA 2006b). The authorisation of placing on the market of this GM potato was annulled by the Court of Justice of the European Union in December 2013 (General Court of the European Union (2013)).

The following second generation plant traits are relevant for potential risks arising from contamination of the food production chain: GM plants used as bioreactors like glucose isomerase producing plants, GM plants producing commodities for industrial purposes like erucic acid or modified starch, GM plants producing substances simplifying industrial processes (e.g. heat-stable enzymes), GM plants with compositional modifications improving its use as feedstock (e.g. reduced lignin content).

If such GM plants are to be commercialised and released into the environment, they need to be adequately and thoroughly risk assessed. The most important issue for the risk assessment of such GM plants is the avoidance of plant material used for production of food/feed commodities getting commingled with GM plant material produced for non-food/feed purposes. This must be controlled strictly by appropriate monitoring measures and physical separation of material and substance flows. In addition to that, the EFSA GMO Panel mentions an obligation for applicants for taking account of confinement measures. This means that cultivation of GM plants solely for technical applications must be clearly separated from agricultural systems for food and feed production. Also, accidental intake by humans, livestock and wildlife animals, exposure of farmers and workers handling the GM plants, and exposure of people living in the vicinity have to be taken into consideration (EFSA 2009b).

Erucic acid and its derivatives are important renewable commodities for industrial applications (e.g. production of lubricants, cosmetics). High levels of erucic acid in cooking and salad oil extracted from rapeseed have been associated with health problems (Nath et al. 2009). Therefore, the level of erucic acid in edible oils and fats must not exceed 5%. This maximum level in order to protect consumers from possible toxic effects was established 38 years ago by the European Commision (EEC 1976). The Austrian regulation (Erucasäureverordnung) is similar (BGBl. Nr. 468/1994).

Although, there have been no confirmed reports of erucic acid causing health problems in humans, health affects have been observed in animals which means that such effects cannot be ruled out in humans. It is, furthermore, possible that frequent consumption of high levels of erucic acid may add to the risk of developing heart disease. For example, incidences of erucic acid exceeding legal limits in some pickles, sauces and preserved vegetables imported to the European Union (United Kingdom) were reported (Food Standards Agency 2004).

Production of thermotolerant enzymes in transgenic plants provides advantages for industrial uses. Primarily, such enzymes are useful to speed up degradation processes of polysaccharides like starch or cellulose without the need of adding bacterial enzymes. But, enzymes that are stable at high temperatures also pose the risk that they are not degraded at food and feed processing steps like roasting or cooking. In case of a thermotolerant α-amylase produced in GM maize 3272, EFSA (2013a) considered that owing to the stability of the newly expressed amylase protein even at high temperatures additional scientific data for safety assessment need to be submitted. Lack of such data resulted in the statement that EFSA could not conclude on the potential for de novo allergic sensitisation (EFSA 2013a). This leads to the conclusion that as the stability of transgenic proteins under relevant processing conditions is an important criteria for demonstrating the safety of transgenic proteins EFSA (2011a), GM plants expressing thermotolerant enzymes can make toxicological and allergenicity assessments more complicated.

4 Developments and trends

As reported in Chapter 2.2, many second generation GM plants are in the phase of research and development, or in the testing phase. Although authorisations are currently worldwide still limited, it seems to be only a matter of time - because of the high marketing potential of newly developed GM plant traits - for entering the market of a number of second generation GM plants.

This chapter discusses if there are certain trends for food production and for the industry. It is not the goal of this chapter to analyse economic or other drivers of the trends for second generation GMO. Based on the field trial information of recent years, what can be derived is where researchers have laid their focus, and what may be expected for authorisation pipelines in the next years - but not on a specific event, only on the traits. The consumer benefits from the traits are described in Chapter 3.2.

4.1 Methodology

The aim of the trend analysis is to illustrate the temporal changes in second generation GM plant field trials regarding plant traits and to derive developments of the near and midterm future. The methodological approach was to make a temporal analysis of the data.

In the year 2003, a trend analysis concerning GMO field trial notifications was performed by a research team (Lheureux et al. 2003). Based on the annual number of field trials, the published report presented a general trend curve as well as figures showing the evolution of specific traits.

In order to provide more details on the field tested GMO traits, the approach used by Lheureux et al. (2003) was modified and not only the "annual number of field trials" but the "annual number of tested traits" evaluated. This means that if two (three, four, five …) traits were tested in one field trial, those were counted as two (three, four, five …) data entries. This would always be the case if GMOs with stacked traits were field tested. Hence, the 473 EU- and the 3777 USA-field trials produced 777 and 5024 data entries, respectively. This means that 777 second generation traits were actually tested in the EU (from 1991 to 2013) and 5024 in the United States (from 1988 to 2013).

This annual number of traits tested (entries) in the EU and the USA (results see Chapter 3) was used for establishing trends for second generation GM plant traits. The data derived from the web databases include field trial data of 26 consecutive years (from 1988 to 2013) providing a good fundament for a trend analysis.

The analysed trends give an indication of the development of each trait and illustrate temporal changes, and can also be used to predict possible future developments. Predictions, however, have to be interpreted with caution due to the fact that the data depend not only on the research goals, but also on other factors many of which are difficult to account for (political decisions, socio-economic factors, etc.).

The focus of the trend analysis was laid on the USDA database, as the European field trial database (JRC) shows a general downward trend since 2003 (Figure 1) which seems to be not trait-related but rather related to external factors (e.g. "de facto moratorium on GMOs" (Pew Initiative on Food and Biotechnology 2005)). Thus, the illustration of the temporal development of EU field trial data results in a downward trend. An analysis of the single traits was not established, since for most of the traits this analysis would not produce significant results with respect to future developments. Moreover, the United States field trials are highly relevant for the future pipeline of GMO, and also notifications in the EU are expected for most GM plants developed for the US market (Lheureux et al. 2003).

4.2 Temporal developments of field trials and approvals

The data derived from the web databases in Chapter 3 were used to make further analyses regarding temporal developments and trends of tested traits of second generation GM plants. The total number of traits tested (entries) in the field trials in the EU are 777 and in the United States are 5024.

Figure 1 shows the temporal development of the field trials and the tested traits in the United States from 1988 to 2013. In the first year, one field trial was carried out for one trait. Later, stacked events were tested in field trials, and therefore the number of tested traits is higher than the number of the field trials. In the year 2005, the number of notified field trials in the United States peaked at 269. Although the number of field trials has fallen by more than a third since, the numbers of tested traits has remained stable in the range between 250 and 360.

Figure 1 also shows the temporal development of the field trials and the tested traits in the European Union. Both EU curves lie well below the US curves. The EU curves show a maximum peak in the year 1996 (106 traits tested) and another (lower) peak in the year 2003 (60 traits tested) and a decline since then. In the last five years, the numbers of second generation GM plant traits tested range between 17 and 7! This development is an indication that in the last 10 years a number of biotechnology companies have shifted research and testing activities outside of the European continent to e.g. the United States, and thus the number of EU field trials and traits tested cannot provide significant data enabling to analyse current or future developments (e.g. authorisations). Therefore, the trend analysis focusses mainly on the US field trial data.

Note that all Figures and Tables refer only to second generation GM plants.

Figure 1: Number of field trials and tested traits in the United States and the European Union from 1988 to 2013

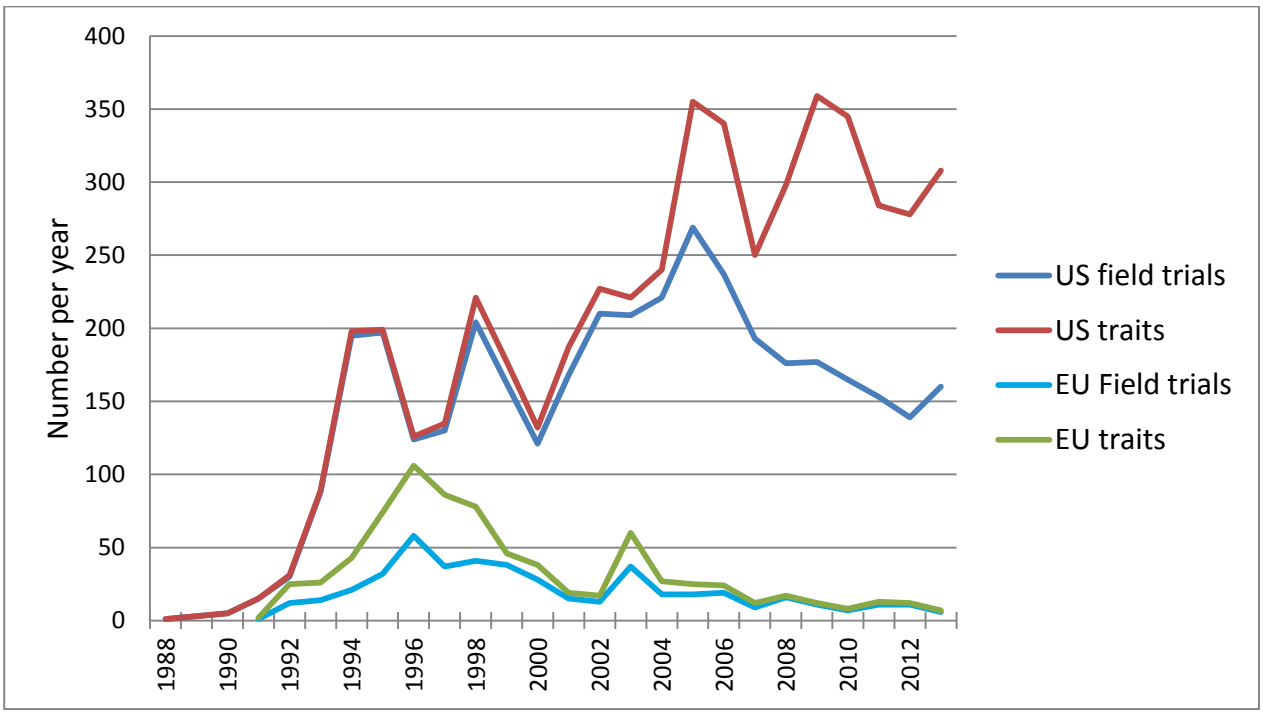


Figure 2 shows the development of the approvals of second generation GM plants in the United States from 1988 to 2013. The figure shows peaks in 1994 and 1999 with seven approvals. Table 5 provides some detail information on the United States approvals.

Figure 2: Number of approvals in the United States from 1988 to 2013

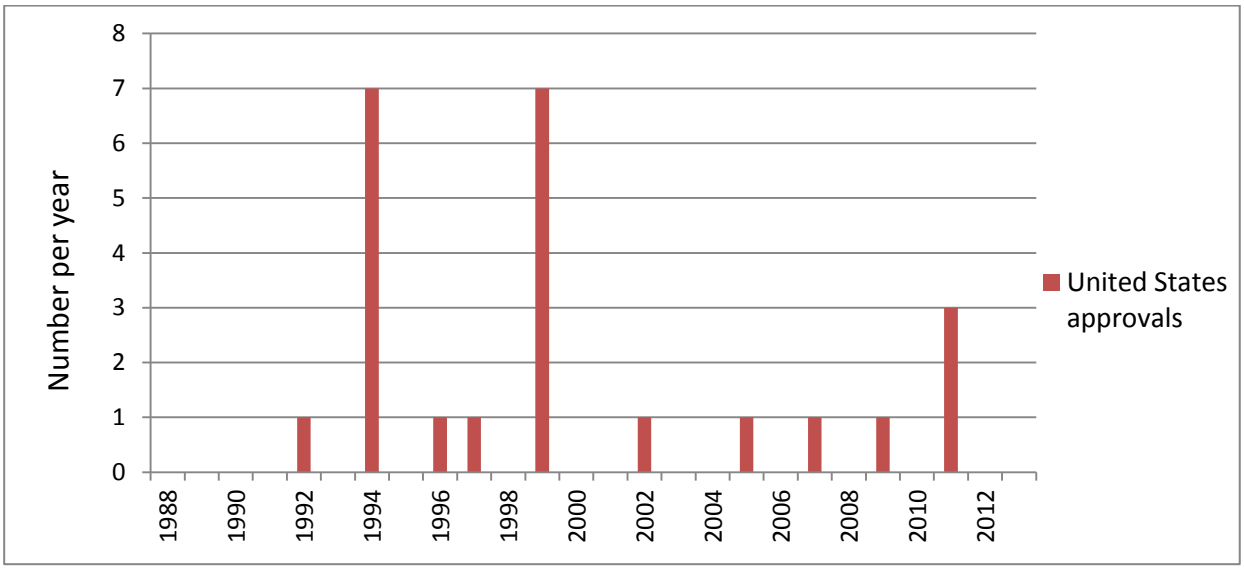


Table 5: Approvals in the United States sorted by year

Plant	Year	Category	Sub-Category
tomato	1992	storage	fruit ripening altered
oilseed rape	1994	commodity	fatty acid
oilseed rape	1994	commodity	fatty acid
tomato	1994	storage	fruit ripening altered
tomato	1994	storage	fruit ripening altered
tomato	1994	storage	fruit ripening altered
tomato	1994	storage	fruit ripening altered
tomato	1994	storage	fruit ripening altered
tomato	1996	storage	fruit ripening altered
soybean	1997	nutrient/ingredient	fatty acid
oilseed rape	1999	anti-nutrient	phytate
oilseed rape	1999	anti-nutrient	phytate
oilseed rape	1999	anti-nutrient	phytate
oilseed rape	1999	anti-nutrient	phytate
oilseed rape	1999	anti-nutrient	phytate
melon	1999	storage	fruit ripening altered
melon	1999	storage	fruit ripening altered
tobacco	2002	nutrient/ingredient	nicotine
maize	2005	nutrient/ingredient	amino acid
maize	2007	processing	protein altered
soybean	2009	nutrient/ingredient	fatty acid
maize	2011	agriculture	stress tolerance
soybean	2011	nutrient/ingredient	fatty acid
soybean	2011	nutrient/ingredient	fatty acid

4.3 Trends of categorised traits

Figure 3 shows the temporal development of the traits divided into seven categories (For further information on the categorisation of the traits please see Chapter 3).

It is significant that the category "nutrient/ingredient" shows a peak in the year 2005 with 172 entries. This number, at this time, counts for about one half of all traits. Together with the category "agriculture", which has its peak also in 2005 (69 entries), it is responsible for about two thirds of the traits. From 2007 on, field trial activities shifted more to the category "commodity", which is in the year 2013 responsible for one fourth of the tested traits. The category "processing" also shows an increase for the last three years indicating that there could be raising interest starting with 2013.

Figure 4 shows information on the categorised traits approved in the United States. The following correlations between field trials and approvals can be observed:

GM plants with traits belonging to the category "storage" were approved in the year 1994. In that year, the number of traits tested in field trials for this category peaked with 51 entries.

One GM maize with increased stress tolerance belonging to the category "agriculture" was approved in the year 2011. In the year 2005, the number of traits tested in field trials for this category peaked with 69 entries.

Figure 3: Number of categorised traits tested in field trials in the United States

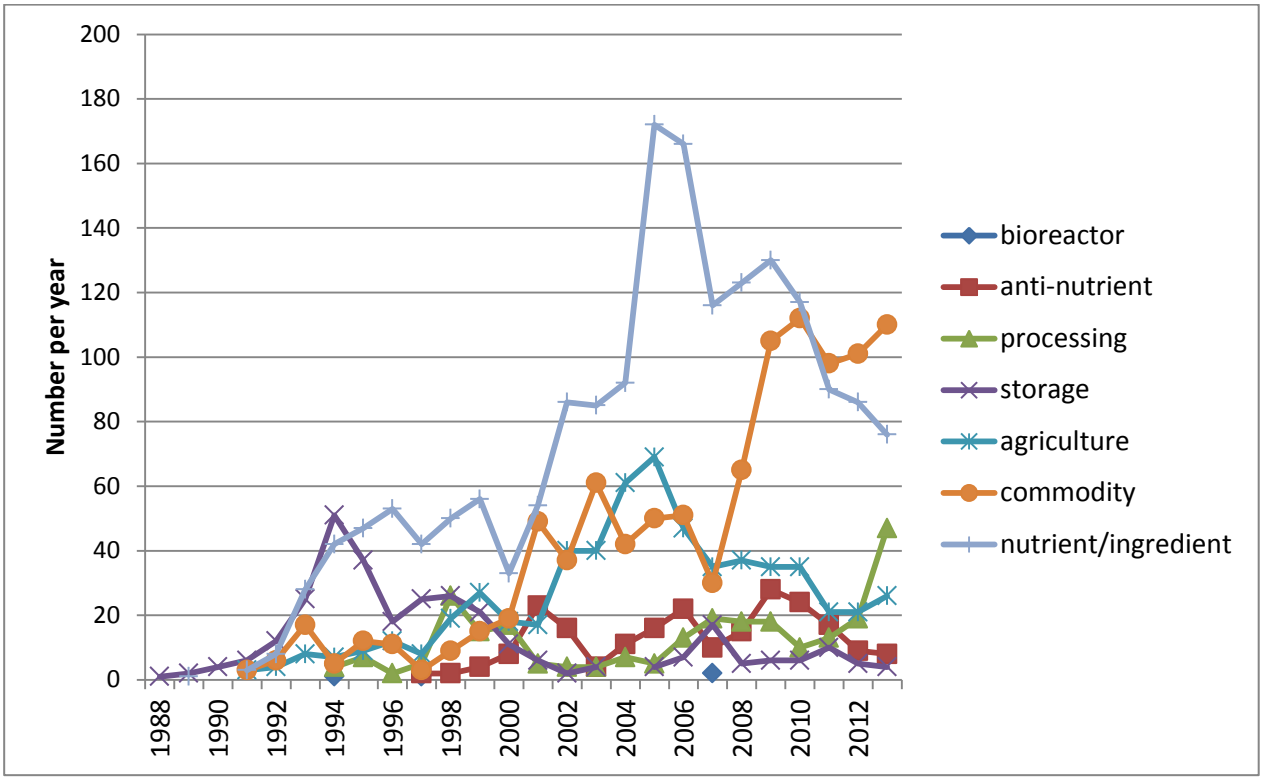
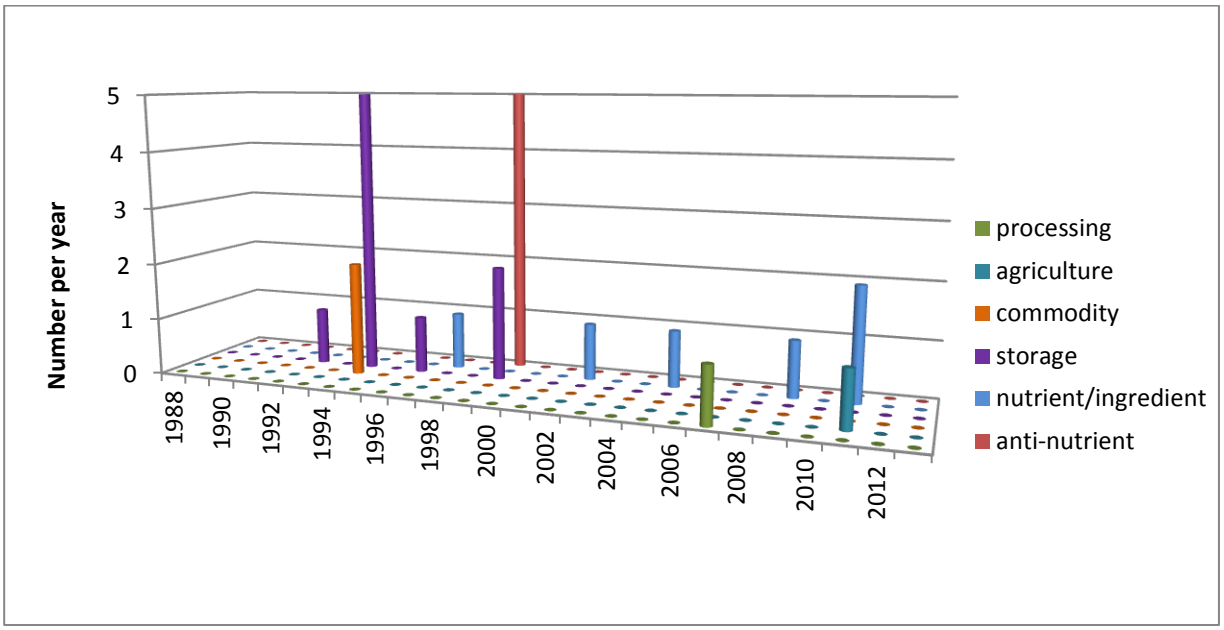


Figure 4: Number of categorised traits approved in the United States



Four of six GM plants with traits belonging to the category "nutrient/ingredient" were approved between 2005 and 2011. This is soon after the number of traits tested in field trials for this category showed maxima of 172 and 166 entries in the years 2005 and 2006.

The number of traits belonging to the categories "commodity" and "processing" increased in the last years. According to these increased activities, a higher interest for commercialisation is expected for the next years.

These results indicate that correlations between GM plant traits tested in field trials and approvals of GM plants exist, in so far as approvals are to be expected in time periods close at or soon after times with maximum values and peaks for the numbers of traits tested in field trials. A similar correlation pattern between field trial data and time of approvals was noticed by Lheureux et al. (2003). These correlations can be shown only at a higher number of traits tested in field trials, which indicates a higher economic interest in application of these traits.

With smaller numbers no correlation can be derived between the traits tested and possible approvals. This is the case for traits belonging to the categories "commodity" and "anti-nutrient": Approvals of GM plants with traits belonging to "commodity" were granted in 1994 and approvals of GM plants with traits belonging to "anti-nutrient" were granted in 1999. In both cases no special peak in field trial activities relating to these categories can be observed around this time.

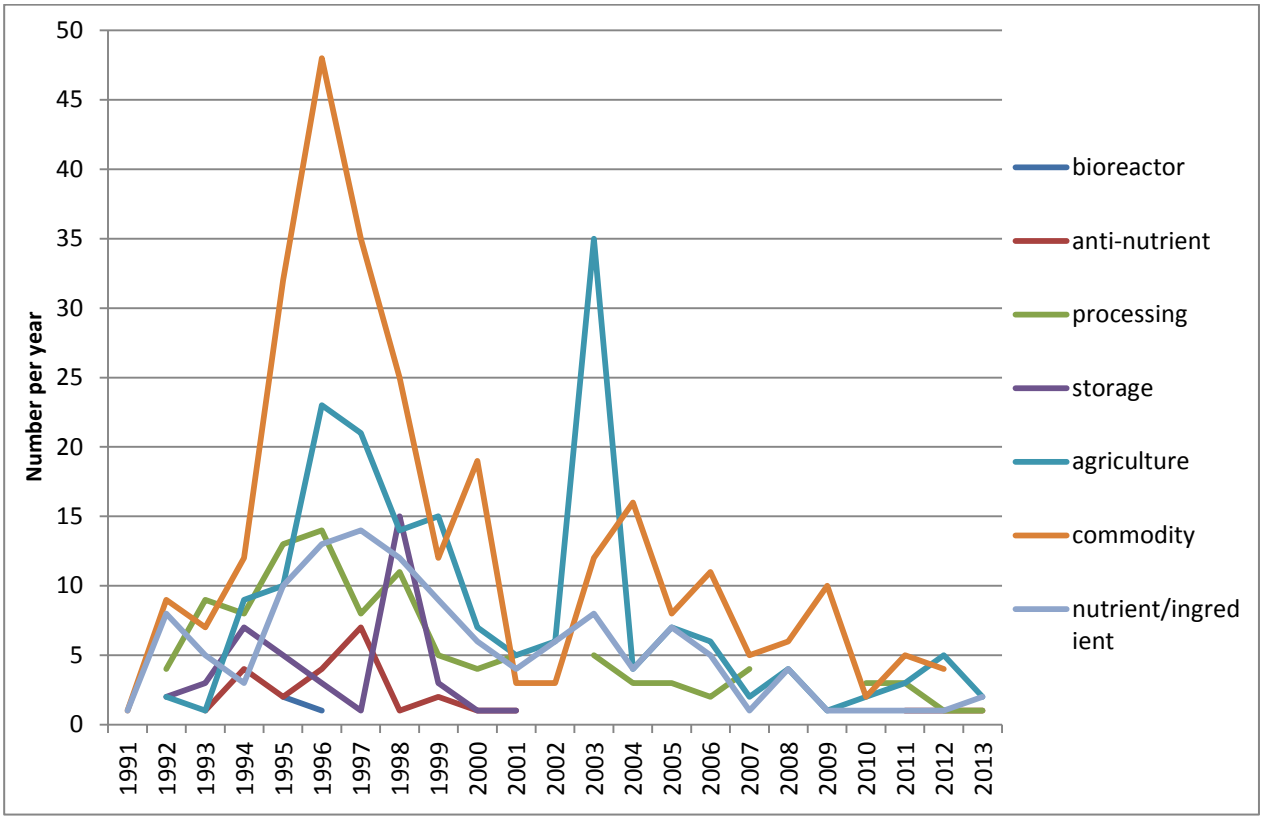
It is assumed that traits with higher economic interest are tested more often and additional tests are necessary for application processes. Therefore, for higher entry numbers in the field trial databases higher probabilities of being commercialised can be expected.

In comparison to the United States, the EU field trial data show a different picture (Figure 6). Maximum peaks occur for the category "commodity" in 1996 (48 entries) and for the category "agriculture" in 1996 and 2003 (23 and 35 entries). "Commodity" mainly relates to tests with GM potatoes with increased amylopectin content, and "agriculture" mainly relates to GM plants with drought tolerance traits.

It is also noticeable that GM plants with traits belonging to the categories "anti-nutrient", "storage", and "bioreactor" have not been field tested in the EU for more than 10 years.

As said before, as there is a general decline in the number of traits tested in the EU since 2003, the EU data are not further discussed or analysed.

Figure 5: Number of categorised traits tested in field trials in the European Union



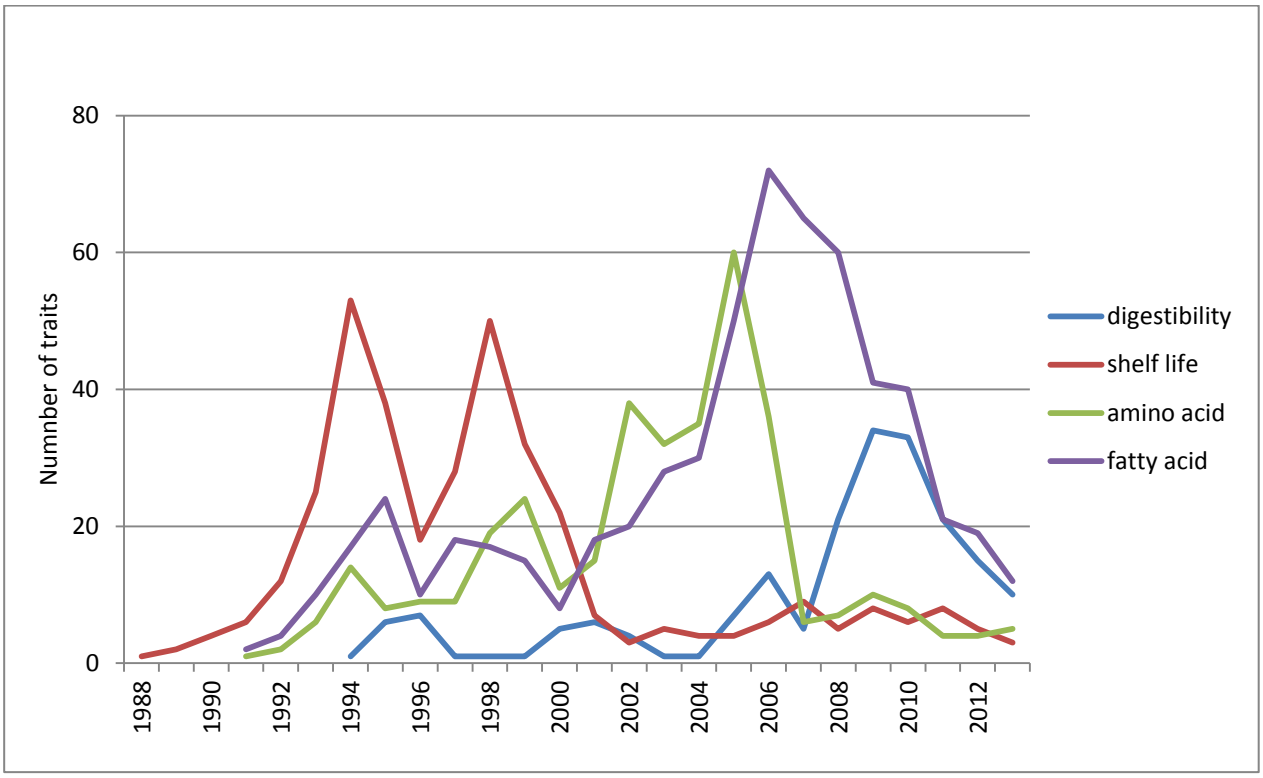
4.4 Trends of subgroups

For a better illustration, similar traits of the USA field trial data were assigned to 17 subgroups, if possible. Of the 5024 entries 2063 could be assigned to subgroups. 2961 entries could not be assigned to a specific subgroup, as the trait names were very vague including names as "altered amino acid", "altered food quality", "altered processing characteristics", etc. (For further information on the assignment of the traits please see Table 29 and Table 30 in Annex).

The four major subgroups included 1522 of 2063 entries and are shown in Figure 6. The subgroup "amino acid" (relating to enhancement of essential amino acid levels) shows a peak in the year 2005 with 60 entries, but within two years the number falls below 10. The subgroup "fatty acid" (relating to changes in fatty acid pattern) shows a peak in 2006 with 72 entries, but the number decreases significantly from that year on. The subgroup "shelf life" (relating to "fruit ripening altered/altered ethylene metabolism/ ethylene production reduced") peaks in the years 1994 and 1998 and also shows a clear decline since then. The decline of these three subgroups seems to point out that the past field trials experiments provided the information needed and that research has now been completed for these traits in the United States (Lheureux et al. 2003). Applications in the area of two of these groups of plant traits can be expected for the near future.

The subgroup "digestibility" (relating to traits like "reduction of anti-nutrients/altered fibre quality/phytate decreased") shows a peak in the period 2009-2011 indicating that for these traits approval activities can be expected for the next years.

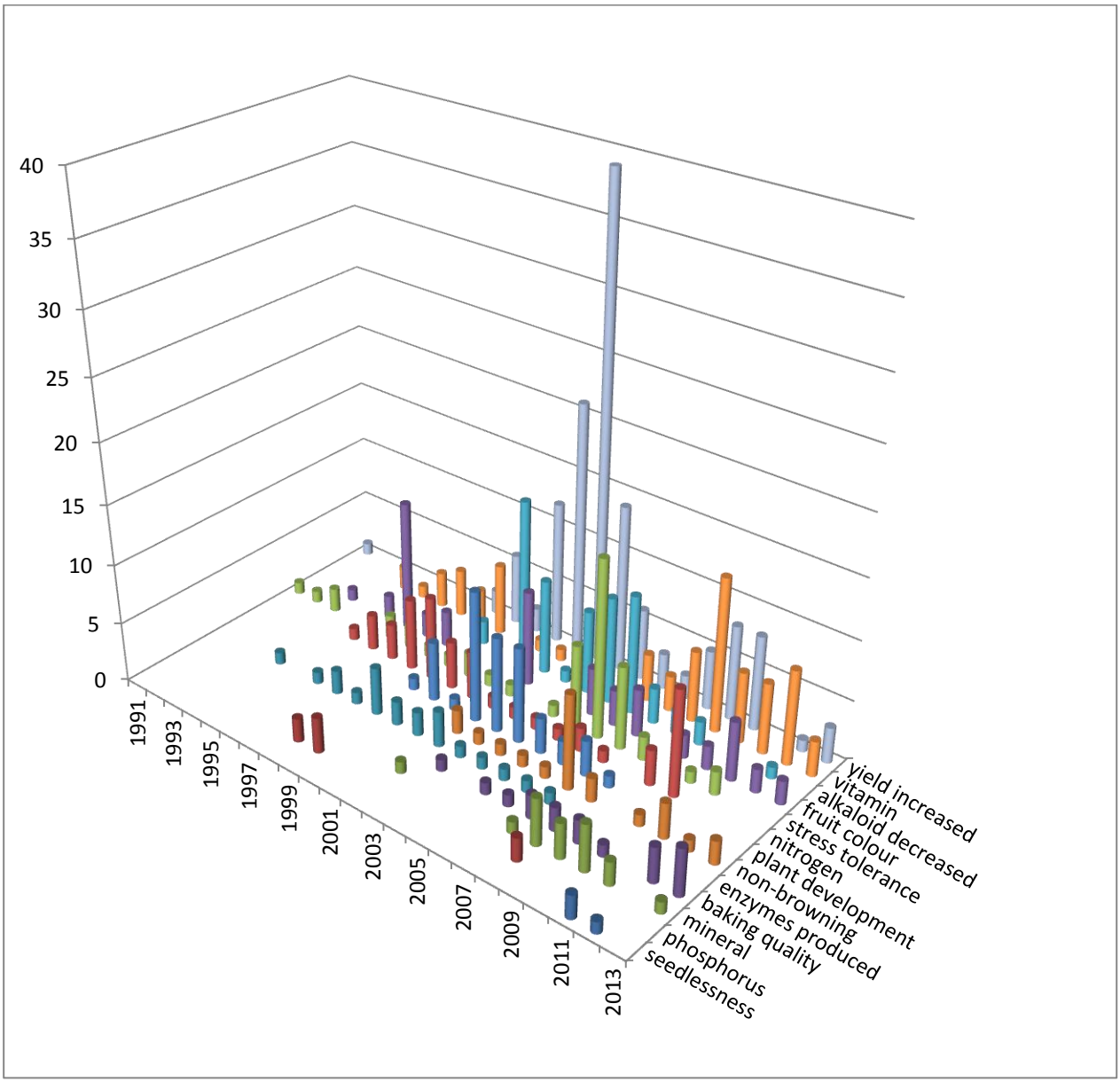
Figure 6: Number of traits tested in field trials in the US of the four major subgroups



Further subgroups representing 541 entries (traits tested) are shown in Figure 7. There seem to be a "window of interest" for several subgroups where field trials and the respective research are done. Traits as "increased yield" and "plant development" had this window of interest between 2000 and 2005.

At least for four subgroups increased number of field trials occurs for the last five years. These subgroups are: vitamin, mineral, baking quality and seedlessness. This currently high relevance for field research in the United States suggests that GM plants with those traits are of high interest for future GMO approvals. The concerned traits are: Plants with enhanced vitamin (pro-vitamin) content, plants with increased mineral content, plants with alterations of gluten protein or enhancement of amylases, and also fruits with increased seedlessness. All of these plant traits yield potentially high consumer benefits.

Figure 7: Number of traits tested in field trials in the US of 13 further subgroups



4.5 Appearance of traits

A further analysis regarding the appearance of traits of both the EU and the USA data (JRC, USDA) uses a methodology of evaluating the first appearance of the traits in the datasets, according to the notification details. Detailed information is presented in Table 31 and Table 32 (see Annex, Chapter 9.3).

Five traits were tested in the EU field trials that appeared in the year 2009 and later indicating a high level of currentness. These traits are expected to be further tested in the near future. 25 traits appeared in the year 2004 and later in the EU field trials indicating a medium level of currentness.

33 traits were tested in the USA field trials that appeared in the year 2009 and later indicating a high level of currentness, at least for the US. These traits are expected to be further tested in the near future. 59 traits appeared in the year 2004 and later in the USA field trials indicating a medium level of currentness.

There are some similarities for two GM plant traits providing direct consumer benefits between European Union and United States field trial appearances that seem to be worth looking at:

- GM plants with increased vitamin content appeared in the year 2009 and later in both field trial databases, with the exception of GM plants with increased vitamin E (tocopherol) content, which first appeared in the US database in the year 2007.
- GM plants with low content of coeliac-toxic epitopes appeared in the year 2009 and later in both field trial databases.

For vitamin enhanced GM plants, the focus of interest in the EU is to more easily produce, extract and purify vitamins for using them as food additives or supplements. The development of GM plants (wheat) with altered gliadin or glutenin proteins brings advantages for people affected by coeliac disease.

These two consumer benefit traits have started field test phase within the last five years, either in the United States or in the European Union. After a phase of evaluation - of the information derived from the field trials - applications for commercialisation of GM plants with such traits could be expected in the next years.

4.6 Conclusions of developments and trends

The trend analysis illustrates temporal changes in second generation GM plant field trials regarding plant traits in the years between 1988 and 2013. The focus was laid on the US data, because the EU field trials show a downward trend since 2003 and would not produce significant results with respect to future developments.

From the analysis and the statements made in the subchapters above, authorisation activities for the categories "commodity" and "processing" in the next years in the United States, and also for the subgroups "digestibility", "vitamin", "mineral", "baking quality" and "seedlessness" are expectable.

For the category "commodity" the major traits concerned are: "altered fatty acid content/altered oil content", "altered protein content", and "decreased lignin content".

For the category "processing", the major traits concerned are "glutenin increased/altered bread making characteristics", "altered grain processing", and "altered biomass processing".

It can be assumed that authorisations in the United States may subsequently lead to proposals for application of the same GM plants in the European Union.

5 Risk assessment of second generation GM plant traits

5.1 Introduction

GM plants of the second generation are developed and produced to provide direct consumer benefits and advantages for industrial processes.

The focus of this chapter is on gaining a deeper insight into the risk assessment of selected second generation GM plants. Challenges and problems associated with GM plant risk assessment are addressed and, specifically, issues in relation to selected second generation GM plant traits highlighted.

The following second generation GM plant traits were selected:

- GM plants with increased oleic acid content
- GM plants with high amounts of stearidonic acid
- GM plants with a very low content of coeliac-toxic epitopes
- GM plants enhanced vitamin content
- GM plants expression of thaumatin
- GM plants producing thermotolerant enzymes
- GM plants with high amounts of lysine
- GM plants increased erucic acid content
- GM plants with improved yield

For four of the abovementioned second generation GM plant traits, notifications have been submitted for different commercial purposes (e.g. processing, cultivation) in some countries:

- GM plants with high amounts of lysine/methionine (event LY038)
- GM plants with increased oleic acid content (events 305423, MON87705)
- GM plants with high amounts of stearidonic acid (event MON87769)
- GM plants producing thermotolerant enzymes (event 3272)

A number of risk assessment documents are available for these five GMO events. The most important scientific statements and documents on the risk assessments of the national risk assessment agencies and national authorities are presented in this report. Additional aspects are discussed.

It should be noted that the Austrian comments on these events published in Annex G Documents can be found at EFSA's Applications Helpdesk (EFSA 2014a).

National risk assessment agencies which made statements on second generation GM plant events mentioned in this report are:

1) the U.S. Food and Drug Administration (FDA), which is the regulatory authority responsible for the safety and post market compliance of food derived from GM plants in the United States,

2) the U.S. Department of Agriculture (USDA), which oversees safety for cultivation & protection of U.S. agriculture/environment,

3) the Canadian Food Inspection Agency (CFIA), which is responsible for the enforcement of the standards and policies that relate to safety and nutritional quality of novel foods in Canada (this includes food derived from GM plants),

4) Food Standards Australia New Zealand (FSANZ), which is a bi-national authority and risk assessment agency developing and administering the Food Standards Code for Australia and New Zealand which lists requirements for foods including GM foods,

5) the Japan Biosafety Clearing House (J-BCH), which is responsible for the enforcement of the Law concerning the Conservation and Sustainable Use of Biological Diversity through regulations on the use of living modified organisms (Cartagena Protocol domestic law) in Japan, and

6) the Philippines Biosafety Clearing-House (BCH-Pilipinas), which provides access to the country's information on GMOs and contains, among others, the National Contacts, laws and regulations on LMOs and the risk assessment of approved regulated articles in the Philippines.

Method used for the exposure assessment

For the exposure assessment, food consumption data were taken from the Austrian food consumption database of the "Austrian Nutrition Report 2008" (Elmadfa et al. 2009). The food intake was assessed using a 24-h recall in adults and an estimated three-day dietary record in children.

For this project on second generation GM plants, the exposure assessment was performed for men and women (19 - 65 years) and children (6 - 15 years). Mean and 95th percentile values were calculated over the collective data from men, women and children. The "collective" is defined as the mean intake of food based on the data collection.

The operating figures for body weight of the Austrian population are for men 81.5 kg, for women 63.3 kg, and for children 39.7 kg.

Values like, for example, fatty acid profiles in several oils, or the water content in maize products derive from the nutritional.software v1.31.30 (nut.s), which is based on the "Österreichische Nährwerttabelle" ÖNWT (Austrian Nutritional Values Table) and the "Bundeslebensmittelschlüssel" BLS (Federal Foodstuffs Key). The ÖNWT is released from "dato Denkwerkzeuge" in cooperation with the Department of Nutritional Sciences Vienna. The BLS is a database which contains the mean nutritional values of about 15,000 food products marketed in Germany. It is a standard tool to analyse nutritional epidemiological studies and food consumption in Germany and was developed by the Max Rubner Institute. The ÖNWT complements the BLS, and hence the software nut.s is a combination of the two databases. It is recommended by the Department of Nutritional Sciences Vienna as basis for all aspects of calculating nutritional values in food industry, canteen kitchens, with caterings, for nutritional consultations and all other aspects in nutritional sciences concerning this matter (ÖNWT 2014).

In the exposure assessment two scenarios are calculated, a maximum intake scenario and a minimum intake scenario. For a "maximum intake scenario" it is considered that 100% of the consumed food is genetically modified. For a "minimum intake scenario" it is considered that 0.9% of the food is genetically modified. (The results presented in the tables of the Chapters "Exposure Assessment" are rounded.)

For GM plants authorised for food and feed purposes in the EU, a 0.9% of GM content in products is determined in Section 2 ("Labelling") in Regulation (EC) No 1829/2003 on genetically modified food and feed, *"This section shall not apply to foods containing material which contains, consists of or is produced from GMOs in a proportion no higher than 0.9 per cent of the food ingredients considered individually or food consisting of*

a single ingredient, provided that this presence is adventitious or technically unavoidable" (EC 2013a). This 0.9% represents the accidental intake of authorised GM material for non-target groups. In both intake scenarios, the highest level found by analysis of any newly produced substances or increased nutrient (e.g. β-carotene, thaumatin) is used in the exposure assessment, because the interest of inventing GM products is to express substances in high amounts.

5.2 GM plants with increased oleic acid content

Enhancement of oleic acid levels in GM plants is a trait that is known to have already been introduced into soybean plants for commercial use. Several countries have authorised high oleic acid soybeans for different scopes including cultivation and food and feed purposes. Experiments for genetic modification of the oleic acid levels in plants have also been carried out for other oilseed crops as e.g. cotton, oilseed rape and false flax (Liu et al. 2002; Jung et al. 2011; Kang et al. 2011; USDA 2014).

The underlying principle of the genetic modification of oilseed crops with high oleic acid content is an inhibition of the *fad2-1* gene encoding a fatty acid ω-6 desaturase that desaturates oleic acid to linoleic acid. Thus, in high oleic acid GM event 305423, the transcription of an inverted repeat (antisense segment) of the *fad2-1* gene (or gene fragment) acts to suppress transcription of endogenous ω-6 desaturase via dsRNA molecules. This gives rise to the high oleic phenotype of GM soybean (Pioneer Hi-Bred International Inc. 2007; EFSA 2013b).

Soybean event MON87705 contains an additional transgenic antisense segment of the *fatB1 gene* suppressing the transcription of endogenous palmitoyl acyl carrier protein thioesterase. The palmitoyl acyl carrier protein (ACP) thioesterase terminates the fatty acid biosynthesis cycle to palmitic acid. Therefore, suppression results in a decrease in the transport of the saturated fats out of the plastid retaining their availability for desaturation to oleic acid (USDA 2011; Canadian Food Inspection Agency 2012).

5.2.1 Molecular characterisation

The characterisation of the transgenic constructs both in event MON87705 and event 305423, and also the three GM soybean sublines G94-1, G94-19 and G168 (derived from transformation event 260-05 (USDA 1997)), which have not been notified in the EU, revealed the insertion of anti-sense sequences from the first intron of the soybean *fad2-1a* gene. Additionally, the insertion of the soybean *fatb1* gene was verified in event MON87705. In event MON87705, a codon-optimised CP4 epsps coding sequence from *Agrobacterium* sp. CP4 was inserted making this GM event tolerant to glyphosate-containing herbicides (EFSA 2012c). In MON87705, the expression of RNA containing an inverted repeat of the *fad2-1a* and *fatb1-a* gene fragment was proven by PCR-based assays. Putative ORF amino acid sequences were tested using bioinformatic analysis and also there was no indication of the disruption of known endogenous soybean genes. Decreases in fad2-1a and fatb1-a mRNA levels in soybean MON87705 seeds were detected by northern blot analysis (FDA 2011; EFSA 2012c). EFSA concludes soybean MON87705 contains a single insert and the putative ORFs do not indicate hazards, and because of the data available (expression, stability test) the molecular characterisation does not raise safety issues (EFSA 2012c).

In 305423, seven copies of the *gm-fad2-1* gene fragment were found to be present in the plant genome. Also, all putative ORF amino acid sequences were tested using bioinformatic analysis, and expression and stability analysis conducted. Northern analysis indicated an effective suppression of transcripts of the endogenous *fad2-1* gene. The inserted DNA (seven gene copies) is genetically linked and segregates following a typical pattern of Mendelian inheritance expected for a single, genetically linked insertion locus. The molecular characterisation of 305423 soybean does not raise safety issues, according to EFSA (2013b).

Risk assessment authorities of other countries conducted similar tests for molecular characterisation of high oleic soybean events. FDA (2009) also evaluated soybean 305423 by Southern blot and sequencing analyses of the DNA inserted, chi square analysis of trait inheritance data and tests of genomic stability were carried out. For event MON87705, the applicant concluded from the bioinformatic analyses that, even in the highly unlikely occurrence of translation of any of the ORFs, the polypeptide products are unlikely to exhibit allergenic, toxic, or otherwise biologically adverse properties (Pioneer Hi-Bred International Inc. 2007). This argument was followed by FDA (2009).

For the Australian and New Zealand authorities the nature of the genetic modification, the molecular characterisation, the characterisation of the novel protein was also highly important (Food Standards Australia New Zealand 2009a; Food Standards Australia New Zealand 2011e). The Japanese authorities collected the same information on the molecular characterisation as other risk assessment agencies for the nation-wide approved soybean events (305423, G94, G168) (e.g. donor nucleic acid, sequence data on the insert) (Japan Biosafety Clearing-House 2005; Japan Biosafety Clearing-House 2007).

From the authors' point of view, additional studies for testing the stability of the RNAi effects under different environmental conditions and through more than four generations should be conducted. DNA sequencing is also needed to verify whether partial transgenic inserts occur at the transformation site. For proper characterisation of the border DNA, sequencing test should be performed including ≥ 1000 bp of flanking DNA on each side.

5.2.2 Comparative assessment

The comparative assessment includes comparisons between the high oleic GM soybeans and their conventional counterparts (non-GM near-isogenic lines). According to EFSA guidances and current EU Regulation (EC 2013a), the compositional endpoints should be taken from OECD consensus documents on compositional considerations for new plant varieties (EFSA 2006a; EFSA 2011a; OECD 2014). Agronomic endpoints are autonomically chosen by the applicant.

For EU approval of event 305423, 92 endpoints were routinely measured by the applicant and compared with the conventional counterpart. Significant differences were observed for the fatty acid profile (high oleic acid content, reduced PUFA) and the newly expressed proteins which were concluded to be consistent with the objective of the modification. For other compounds showing significances, no further assessment was deemed necessary owing to their well-known biochemical roles and to the magnitude of the reported levels (EFSA 2013b).

For event MON87705, the EFSA GMO Panel considered the total compositional data supplied and the observed compositional differences between soybean MON87705 and its conventional counterpart in the light of the field trial design, measured biological variation and the level of the studied compounds in non-GM soybean reference varieties, and concludes that no biologically relevant differences were identified between soybean MON87705 and the conventional counterpart and other non-GM soybean reference varieties, except for the fatty acid profile and the newly expressed protein CP4 EPSPS (EFSA 2012c).

For the agronomic parameters for both events (305423 and MON87705), the magnitudes of the differences in agronomic parameters were considered small. Also, they fell within the ranges observed for commercial soybeans. The GMO Panel considered these differences to be of no biological relevance (EFSA 2012c; EFSA 2013b).

Events G94 and G168 were also comparatively assessed. The results showed an expected modified fatty acid pattern as compared to the conventional counterpart. Furthermore, the levels of accumulation of two

important seed storage proteins, glycinin and β-conglycinin, were changed. Seed storage proteins ensure normal grain development in plants (Shewry and Halford 2002). The glycinin levels increased, while the β-conglycinin levels decreased. It was concluded that variations in seed storage proteins are common and the protein gel of a released commercial soybean variety (Altona) shows a very similar protein banding pattern to that of the high oleic soybeans.

The detailed information of the fatty acid composition modification and the compositional analyses, including a complete fatty acid analysis, characterise the fatty acid metabolism of the high oleic soybeans. GM soybean oil contains approximately 10% saturated fats, greater than 80% oleic acid, approximately 2% linoleic acid and 3.5% linolenic acid. Trace amounts (0.5%) of a linoleic acid 9,15 isomer were also detected, which is absent in non-hydrogenated soybean oil, but present at similar levels in butterfat, and is often found at considerably higher levels (typically 1-3%) in partially hydrogenated vegetable oils (FDA 1996; Canadian Food Inspection Agency 2011a).

The Australian and New Zealand authorities also considered a compositional analysis and evaluation of nutritional issues as very important (e.g. the question, if food derived from high oleic soybeans is as safe for human consumption as food derived from conventional varieties of soybean). Besides the abovementioned variation in the fatty acid profile, the high oleic soybeans G94 and G168 were found not to differ substantially from control soybeans in levels of amino acids, vitamins, minerals, trypsin inhibitor, phytic acid, the oligosaccharides raffinose and stachyose, daidzein or genistein. Only glycitein and lectin levels were slightly elevated, but it was concluded that they were about in the middle of levels reported in the literature and do not pose any safety concerns (Australia New Zealand Food Authority 2000).

Japanese authorities evaluated comparative data in GM soybeans as well. Significant changes (event G94) were observed for β-conglycinin and glycinin or glycinin precursor. They pointed out that such differences were also observed in soybean varieties improved by crossbreeding methods and mutation induced soybeans, and therefore the changes were considered well within the ranges recognised for conventional soybeans. The Japanese authorities finally concluded that there were no risks that the use of the soybeans G94 and G168 causes adverse effects on the environment (Japan Biosafety Clearing-House 2005).

From the authors' point of view, a comprehensive discussion covering any of the unexpected outcomes of the comparative analysis (e.g. changes in seed storage protein levels) has to be done including detailed analysis in relation to unintended metabolic changes. This analysis should account for the interrelationship among protein, carbohydrate and fatty acid metabolisms with a need to include state-of-the-art literature on natural variations of plant compounds to allow drawing final conclusions on the substantial equivalence of the GM soybeans.

5.2.3 Toxicological and allergenicity assessment

The toxicological risk assessment of event 305423 consisted of a (subchronic) 95-day feeding study in rats. Additionally, efficacy studies with chicken, hens and pigs were conducted. In the subchronic toxicity study, groups of 12 male and 12 female Crl:CD(SD) rats were fed balanced rodent diets from GM soybean 305423 (test group), a negative segregant (control group), and commercial non-GM soybean varieties. All soybean fractions and diets were nutritionally similar, with the expected fatty acids level changes in 305423 oil and diet. Data from animals fed commercial varieties were used to obtain information on the normal range of the examined parameters.

Body weight, body weight gain, feed consumption and feed efficiency, and ophthalmological as well as neurobehavioural evaluations showed no relevant differences between groups. Haematology, coagulation and clinical chemistry analyses showed no statistically significant differences between the test and control group.

Some isolated statistically significant differences between the test and control group were observed: a statistically significant lower heart weight (relative to brain weight) was seen in males fed the test diet in comparison with controls. This was not accompanied by changes in other cardiac endpoints (absolute and relative-to-body organ weight; macroscopic or microscopic findings) and is considered incidental. No relevant differences between groups were seen at macroscopic examination. Microscopic examination was only performed in the test and control group, and the nature and the incidence of the findings were similar and typical for animals of this strain and age.

Because of the use of a negative segregant as the sole control material, the EFSA GMO Panel considers that the repeated-dose subchronic oral toxicity study has limitations for the safety assessment. The use of a negative segregant as the sole control material for comparative assessment does not comply with current EU law (EC 2013a).

For the allergenicity assessment, the applicant performed *in vitro* allergenicity studies with extracts of seeds from soybean 305423 and its conventional counterpart. One-dimensional (1-D) immunoglobulin (IgE) immunoblot analysis as well as ELISA inhibition tests, and also 2-D immunoblot analysis were carried out using pooled sera from individuals allergic to soybean. No meaningful differences in the IgE binding patterns were detected. It was, therefore, concluded by EFSA that the sera from allergic individuals had similar reactivity to proteins in extracts from soybean 305423 and the conventional counterpart, and that there are no indications that the genetic modification might significantly change the overall allergenicity of soybean 305423 (EFSA 2013b).

The authors agree with the conclusions made by EFSA on the lack of significance of the repeated-dose *in vivo* study due to the use of a negative segregant. From the authors' point of view, a 90-day toxicity study should be performed using appropriate control material derived from non-GM conventional counterparts. Furthermore, it has to be demonstrated that the GM plant and its products do not cause potential negative effects only revealed in times of high performance such as reproduction or health stress or long-term influences. It would be also important for the safety of the products derived from high oleic acid GM soybean that the allergenicity studies are complemented with new analytical methods that combine structural information with analysis of conservation of primary structure as described e.g. by Ivanciuc et al. (2011): automated exchange of molecular information on allergens and IgE epitopes between databases and integration of *in silico* tools that are used to investigate allergenicity and cross-reactivity.

5.2.4 Risks associated with pleiotropic effects

The absence of unintended and pleiotropic effects for event MON87705 was confirmed by several tests that were conducted during the evaluation of the different risk assessment agencies. They consisted mainly of flanking sequence analysis, comparative assessment, and toxicity studies with animal models.

Soybean event 305423 showed unintended effects concerning changes in the levels of odd chain fatty acids (C17:0, C17:1, C19:1). It was, therefore, discussed that there appear to be no published studies describing the catabolism of these odd chain fatty acids in mammals (EFSA 2013c) giving indication of existent data gaps in relation to safety aspects of food products derived from second generation GM plants. In spite of the lack of data, EFSA concluded that, although the replacement of vegetable oils with oil derived from soybean 305423

would increase odd chain fatty acids intake, these changes would be small and without impact on health and nutrition.

According to the available data, the following statements were further made by the risk assessment agencies:

FDA agreed with the applicant that with the exception of the intended change in fatty acid composition, the 305423 soybean and the soybean event MON87705 and the foods and feeds derived from it are not materially different in composition, safety, or any other relevant parameter from soybeans now grown, marketed, and consumed in the United States (FDA 2009; FDA 2011). The conclusion on the new soybean varieties G94-1, G94-19 and G168 were similar (FDA 1996). The differences in the levels of odd chain fatty acids were not mentioned by FDA (2009).

The Australian and New Zealand authorities also came to the conclusion that the safety assessment did not identify any public health and safety concerns associated with the genetic modification used to produce high oleic acid soybean lines 305423 and MON87705 (Food Standards Australia New Zealand 2009b; Food Standards Australia New Zealand 2011f).

The comparative assessment for event MON87705 showed no unintended effects, but an intended decreased level of saturated fatty acids (palmitic and stearic acids) and increased level of mono-unsaturated oleic acid and decreased level of polyunsaturated linoleic acid compared with conventional soybean. EFSA concluded that the estimated changes in fatty acid intake resulting from the replacement of conventional vegetable oils with oil from soybean MON87705 do not raise nutritional concerns in the context of the intended use, as specified by the applicant.

However, uncertainties regarding potential unintended effects remain as additional toxicity studies evaluating potential long-term (chronic), reproductive or developmental effects were not conducted. In addition, shortcomings in the risk assessment (e.g. the use of a negative segregant) should be addressed by performing further tests in order to support the conclusions that the final products (food, feed) do not present any health risks.

5.2.5 Exposure assessment

Development and marketing of second generation GMOs are in many cases targeting at substantial modifications of the ingredients content. In this context, the enrichment of nutrients in plants may lead to an oversupply of specific parts of the population not designated as target groups, causing negative effects on the organism. Therefore, the exposure assessment plays a crucial role within risk assessment.

The fatty acid profile of the GM soybean is altered to result in increased oleic acid (C18:1), decreased linoleic acid (C18:2) and palmitic acid (C16:0).

"Soybean MON87705 and all food, feed and processed products derived from soybean MON87705 are expected to replace a portion of similar products from commercial soybean. Oil from this soybean might also replace oils from other sources than soybean. The applicant stated that soybean MON87705 oil is targeted for applications such as margarine, salad dressing, mayonnaise and spread, and home-use liquid vegetable oil, but not for commercial frying (i.e. high-temperature and repeated frying) (EFSA 2012c)."

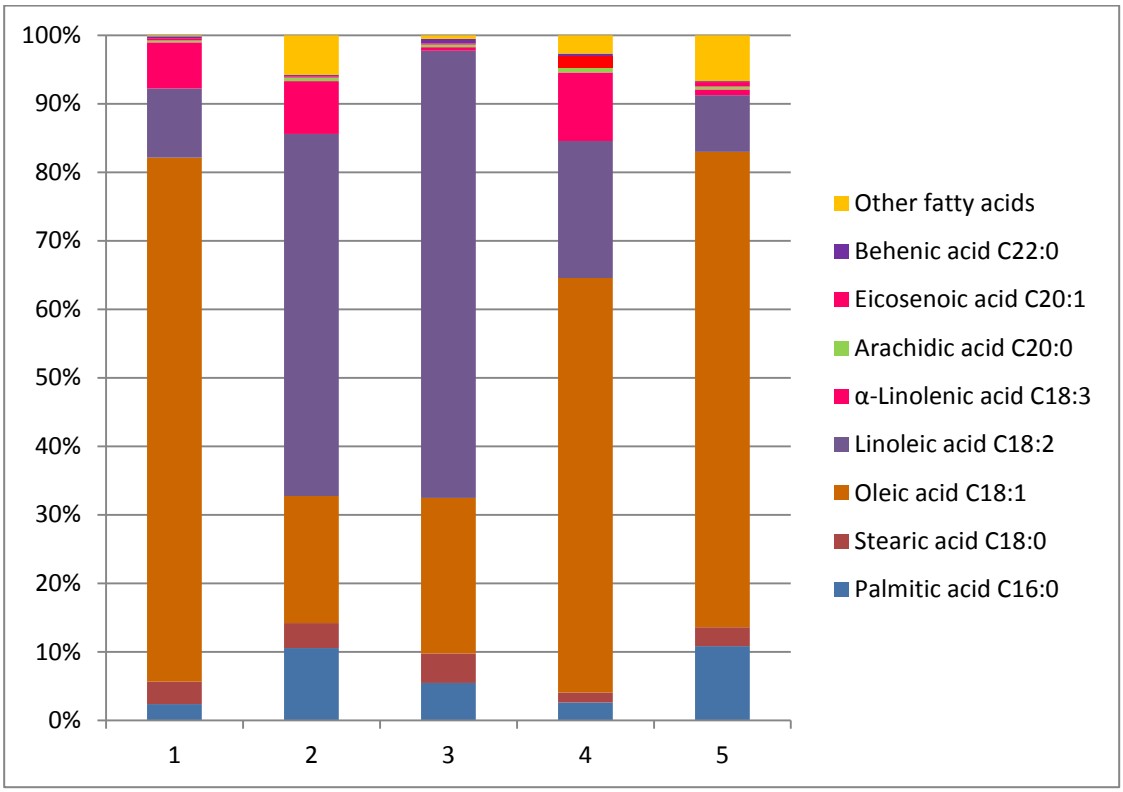
Percentages of fatty acids in GM soybean MON87705 oil compared to (non-GM) conventional vegetable oils (soybean, sunflower, rapeseed and olive) are shown in Figure 8 in the frame of determining the nutritional values of those vegetable oils, concentrating on oleic acid. Results on the fatty acid profile of the GM soybean oil originate from the EFSA Scientific Opinion on application of the genetically modified soybean MON87705

(EFSA 2012c). The fatty acid values for soybean, sunflower, rapeseed and olive oil are derived from nutritional.software v1.31.30 (nut.s) which is based on the "Österreichische Nährwerttabelle" ÖNWT (Austrian Nutritional Values Table) and the "Bundeslebensmittelschlüssel" BLS (Federal Foodstuffs Key). The ÖNWT is released from "dato Denkwerkzeuge" in cooperation with the Department of Nutritional Sciences Vienna. The BLS is a database which contains the mean nutritional values of about 15,000 food products marketed in Germany. It is a standard tool to analyse nutritional epidemiological studies and food consumption in Germany and was developed by the Max Rubner Institute. The ÖNWT complements the BLS, and hence the software nut.s is a combination of the two databases. It is recommended by the Department of Nutritional Sciences Vienna as basis for all aspects of calculating nutritional values e.g. in food industry, canteen kitchens, with caterings, for nutritional consultations and all other aspects in nutritional sciences concerning this matter (ÖNWT 2014).
For the nutritional assessment of the GM soybean MON87705 the increase of oleic acid (C18:1), the decrease of linoleic acid (C18:2), and the decrease of palmitic acid (C16:0) has been taken into consideration. The decrease of stearic acid (C18:0) and α-linolenic acid (C18:3) in MON87705 is only minor compared to the conventional soybean and is not taken into consideration specifically.

The fatty acid profile of rapeseed oil and olive oil, which are commonly used vegetable oils in the EU, in particular the oleic acid content, is similar to that of the GM soybean oil. Both oils contain naturally high amounts of oleic acid and low amounts of linoleic acid; so, this oil composition is not unique to the GM plant but occurs in nature. Even if the replacement of rapeseed and olive oil by the GM product was assumed, no particular exposure assessment of the MON87705 soybean oil would be necessary in our view, due to the fact that these oils are comparable based on the similar fatty acid profile. The *trans* fatty acid content is negligible for uses of this GM soybean oil, like it is for olive and rape seed oil. Increased levels of *trans* fatty acids for uses of the GM soybean oil are not likely compared to uses of conventional, hydrogenated soybean oil.

Moreover, no special consumer groups are discernable for GM soybean oil containing a high amount of oleic acid, as it may be consumed by the whole European population in comparative quantities.

Figure 8: Percentages of fatty acids in GMO soybean oil compared to (non-GM) conventional vegetable oils



1 = GMO soybean in g/ 100g oil (EFSA 2012c)
2 = soybean in g/ 100g oil[1]
3 = sunflower in g/ 100g oil[1]
4 = rapeseed in g/ 100g oil[1]
5 = olive in g/ 100g oil[1]
[1] BLS = Bundeslebensmittelschlüssel (Federal Foodstuffs Key)

In conclusion, it is referred to the statement on the nutritional assessment by EFSA (2012c) that based on an exposure assessment considering the intended use of the GM soybean oil the resulting changes in fatty acid intake (increased intake of oleic acid and decreased intake of linoleic and palmitic acid) do not raise nutritional concerns.

5.3 GM plants with high amounts of stearidonic acid

The enhanced production of stearidonic acid (SDA) in GM plants can be seen as a typical example for the genetically engineering of plants for nutritional purposes. Stearidonic acid is a precursor of the long-chain ω-3 eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Both are essential fatty acids and important for human health, since they are known to reduce the risks for coronary heart disease (Mozaffarian et al. 2005).

The most prominent GM plant with the high stearidonic acid trait is soybean event MON87769, which has been approved in the United States, Canada, Australia, New Zealand, South Africa, Colombia, and Mexico. The first approval was granted in the year 2011. Updated database information shows that this soybean event now has been authorised in two Asian countries as well, South Korea and Taiwan (ISAAA 2014b). The MON87769, currently, is not approved for any commercial purposes in the EU, although a scientific evaluation by EFSA has been published this year (EFSA 2014b).

The underlying principle of the genetic modification of event MON87769 is the insertion of a gene cassette containing the *Pj.D6D* gene encoding a ω-12 desaturase and the *Nc.Fad3* gene encoding a ω-3 desaturase

protein. *Pj.D6D* and *Nc.Fad3* originate from *Primula juliae* (purple primrose) and from *Neurospora crassa* (red mould), respectively. Both genes encode desaturase proteins directly involved in the oleic acid metabolism.

Both newly expressed proteins lead to the accumulation of stearidonic acid in the soybean seed. The ω-12 desaturase increases the production of SDA from α-linolenic acid (ALA), whereas the ω-3 desaturase catalyses the conversion of linoleic acid (LA) to ALA. Since the conversion of ALA to SDA in the human body is slow, consumption of soybean oil derived from MON87769 could avoid this metabolic step. Since stearidonic acid is a precursor of eicosapentaenoic acid these results in a more efficient synthesis of the higher chain-length ω-3 polyunsaturated fatty acids (PUFA) in the body (EFSA 2014b).

5.3.1 Molecular characterisation

The verification of the molecular characterisation of the transgenic construct of event MON87769 as presented by the applicant was verified by different national risk assessment agencies. It consisted of information on the vector system (e.g. donor sequences), a proper description of the intended traits, sequence information of the inserted gene cassette as well as expression data of different parts of the plants. Genetic and phenotypic stability was tested from four generations using Southern blot technique.

It was found that the following important DNA sequences were successfully integrated into the soybean genome (EFSA 2014b):

- Seed-specific promoters of soybean *sphas1* and *sphas2* genes (encoding β-conglycinin) to direct the transcription in the seed
- Coding sequence for the fatty acid ω-12 desaturase from *P. juliae* (primrose) (*Pj.D6D*)
- Coding sequence for the fatty acid ω-3 desaturase from *N. crassa* (*Nc.Fad3*)
- UTR of the *rbcS2* gene that directs polyadenylation of the mRNA
- FMV promoter (P-FMV) from Figwort mosaic virus *35S RNA* gene
- UTR from the *shkG* gene to enhance expression
- Sequence encoding the transit peptide region of *A. thaliana* EPSPS to direct the CP4 EPSPS protein to the chloroplast (*TS-CPT2*)
- Modified coding sequence of the *aroA* gene (encoding EPSPS)

It was also noted by the applicant that the Nc.Fad3 protein of *N. crassa* consists of a single amino acid change (from threonine to alanine) (Food Standards Australia New Zealand 2011d; EFSA 2014b).

The FDA as regulatory authority responsible for the safety and post market compliance of food derived from GM plants in the United States requested Southern blot data for characterising the expression cassette. Furthermore, bioinformatic analyses of the MON87769 soybean insert and flanking genomic DNA sequences were ordered, in order to determine whether insertion of the introduced DNA created potential open reading frames that may encode for a potential harmful protein (e.g. toxin). Alternate reading frames within the coding sequences of the expression cassettes were also tested. No evidence was found that any of the inserted sequences were translated to yield polypeptide products other than intended. The stability of the insert was characterised using Southern blot analysis, and it was tested whether the inheritance of the transgenic DNA follows the expected Mendelian pattern of segregation for a single locus (FDA 2012a).

The data provided to the CFIA were similar to those requested by the FDA and consisted of information on the vector used, the inserted DNA, studies confirming the absence of vector backbone sequences, stability tests in form of Southern blot fingerprints, and protein expression analysis (Canadian Food Inspection Agency 2011c).

The FSANZ received an application for soybean MON87769 in 2010 and - with respect to the characterisation of the genetic material introduced into this soybean event - refers to analogue data: information on the transformation method, vector/plasmid data, insert sequencing and characterisation of the key genetic elements (e.g. function of transgenes), plasmid backbone and ORF analysis, genetic stability and segregation tests (Food Standards Australia New Zealand 2011d; Food Standards Australia New Zealand 2011c).

It was concluded - by all national risk assessment agencies - that one intact single copy of the insert in soybean MON87769 was integrated and maintained across multiple generations. In addition, plasmid backbone sequences were not detected, and no antibiotic-resistance marker genes introduced into the host genome. Stability of the insert was confirmed over at least three generations and Mendelian pattern of inheritance were experimentally proven (Canadian Food Inspection Agency 2011c; Food Standards Australia New Zealand 2011d; FDA 2012a; EFSA 2014b).

In conclusion, the insertion of a single gene cassette was demonstrated by the experiments that were assessed by the national agencies. However, from the authors' point of view, additional data are needed to finalise the molecular risk assessment: Test allowing the identification of even minor changes in the inserted sequences compared to the sequence intended, including detection of small alterations. DNA sequencing is also needed for identification of mutations, DNA rearrangements, translocations, or superfluous DNA insertion in the flanking regions and should include at least 1000 bp of flanking DNA on each side.

5.3.2 Comparative assessment

Comparative field studies for an evaluation of compositional, agronomic and phenotypic characteristics were conducted in the United States in the years 2006 and 2007. The used plant lines consisted of the GM soybean MON87769 (test line), its conventional counterpart (variety A3525, control line), and a number of different commercial non-GM soybean varieties for establishing data on the natural variation of the tested parameters amongst commercial soybean varieties (EFSA 2014b).

For application for commercialisation of soybean MON87769 in the European Union, it was proven by comparative analysis that the newly expressed desaturases in the GMO event led to the intended alteration of the fatty acid profile, especially the high production of stearidonic acid (SDA). Other effects were that in the test line (GMO) oleic acid was slightly decreased, linoleic acid highly decreased, and palmitic acid slightly increased. The levels of stearidonic acid were ranging from 16.88 to 28.35% of the total fatty acid content. Another result of the compositional analysis, however, was that also γ-linolenic acid and two trans-fatty acids (trans-ALA and trans-SDA) were produced in the seeds of soybean MON87769 but not in the seeds of the control lines. Based on the information of the Application Summary (Biosafety Clearing House 2013) these results can be considered to be unexpected. Anyhow, these unintended changes in the fatty acid pattern of the transgenic soybean were evaluated for any nutritional impact and found to be of no biological relevance (EFSA 2014b).

Evaluation of phenotypic and agronomic characteristics (early stand count, seedling vigour, plant growth stages, days to 50% flowering, flower colour, plant pubescence, plant height, lodging, pod shattering, final stand count, seed moisture, 100-seed weight, test weight and yield) revealed that the mean values observed for soybean MON87769 fell within the minimum and maximum mean values estimated for the reference lines. Developmental differences or differences in pollen characteristics (diameter, morphology and viability) were not observed. It was concluded by EFSA (2014b) that no further assessment was deemed necessary.

As well as for the European application, for other national risk assessment agencies like the FDA, the applicant also forwarded results of additional compositional analysis with soybean processed fractions. These studies included the following products:

- defatted toasted soybean meal;
- refined, bleached, and deodorised soybean oil (RBD oil);
- protein isolate;
- and crude lecithin fractions.

The FDA commented the comparative analysis stating that for soybean meal, statistical differences in four amino acids were found, but they were found to be small and the mean and the range of the values were within a tolerance interval for the reference varieties. In RBD oil, *trans*-ALA was statistically higher for MON87769 soybean, but the levels were seen to be comparable to levels of *trans*-ALA in commercial soybean oil and concluded to raise no safety concerns. In protein isolate, a statistically significant (but small) difference in the leucine content was reported, which the applicant considered to be not biologically meaningful. No significances were observed in the phosphatide levels in crude lecithin between the GM soybean MON87769 and the control line (FDA 2012a).

Similar comparative test with soybean event MON87769 were requested by the national food safety authorities in Canada, Australia and New Zealand. The Canadian Food Inspection Agency evaluated the significant findings of the study and concluded that the means and range values of test crop were within the commercial tolerance interval and literature values, and that the trait will not confer to soybean event MON87769 any characteristic that would raise any safety concerns of soybean event MON87769 (Canadian Food Inspection Agency 2011c).

Changes in the fatty acid metabolism were analysed by the Food Standards Australia New Zealand. The catabolism of *trans* fatty acid was examined. These are usually converted following cycles of β-oxidation to the final two carbon product in humans and animals. It was also noted that metabolic pathways for oxidising *trans* unsaturated fatty acids present in the diet are not fully resolved. The conclusions were that minor increases in the levels of most amino acids and total protein were observed, although there are no changes in key constituents in MON87769 seed that represent a safety concern when compared to commercial soybean varieties or other commonly consumed vegetable oils (Food Standards Australia New Zealand 2011d).

From the authors' point of view, the following additional evaluations in case of observed significances are needed and should be carried out:

- better accounting of results of within-site analyses,
- the subsequent application of statistical approaches that yield more statistical power (EFSA 2010c),
- additional field trials for testing further endpoints under different environmental conditions,
- utilisation of new approaches like omics techniques, especially proteomics.

5.3.3 Toxicological and allergological assessment

For the toxicological assessment the applicant conducted, besides *in vitro* tests, acute toxicity studies in which both transgenic desaturase enzymes isolated from soybean MON87769 were administered to mice. No negative effects were observed at doses of 4.66 and 37.3 mg/kg body weight for the PjΔ6 desaturase and the NcΔ15 desaturase, respectively. However, EFSA pointed out that acute toxicity testing of the newly expressed proteins is of little value for the risk assessment of the repeated consumption of food and feed from GM plants by humans and animals (EFSA 2014b).

Furthermore, according to EFSA (2014b), MON87769 soybean oil was also tested in a subchronic toxicity study and a one generation reproductive toxicity study, which were adapted from respective OECD guidelines (OECD 1983; OECD 1998). In the subchronic study, haematology analysis showed statistically significantly higher mean absolute and per cent basophil counts in the high dose group. The absolute basophil counts were similar in the low dose group and the menhaden fish oil control group, and all values were within the ranges of the historical controls of the testing facility. It was concluded by EFSA (2014b) that both feeding studies showed no indications of adverse effects.

FDA also evaluated the different *in vitro* and *in vivo* studies as submitted by the applicant with regard to the toxicological and the allergological risk assessment: homologous searches, digestibility assays, expression profiles, acute toxicity studies with the transgenic proteins, repeated dose animal studies with defatted soybean meal, etc. Based on this information, FDA did not identify any safety or regulatory issues with food ingredients or soybean meal derived from MON87769 soybean that would require further evaluation, and agreed with the applicant that there are no safety concerns from uses of MON87769 soybeans in human food (FDA 2012a).

The Canadian authorities noted that the trait would not confer to soybean event MON87769 any characteristic that would raise any concerns regarding the safety or nutritional composition of soybean event MON87769 (Canadian Food Inspection Agency 2011c). Australian and New Zealand authorities pointed out the animal studies with soybean oil and processed soybean meal showed no adverse effects (Food Standards Australia New Zealand 2011d).

The risk assessment agencies came to the conclusion that the bioinformatics analysis did not reveal sequence homology of the newly expressed proteins with known toxins, that these proteins were rapidly degraded by pepsin *in vitro*, and that humans and animals consume other desaturases daily with no reported adverse effects. Regarding the unexpected modification of the fatty acid pattern (two trans-fatty acids) it was furthermore stated that no adverse effects of these trans-fatty acids are expected when conventional vegetable oils are replaced by MON87769 soybean oil. Moreover, it was concluded the two 90-day dose toxicity studies and the one generation reproductive toxicity study did not induce toxicologically relevant effects when compared with conventional soybean oil (Canadian Food Inspection Agency 2011c; Food Standards Australia New Zealand 2011d; FDA 2012a).

From the authors' point of view, the highest dose level (15% soybean meal in the diet) in the 90-day toxicity study were not chosen in accordance with state-of-the-art requirements: "*Unless limited by the physical-chemical nature or biological effects of the test substance, the highest dose level should be chosen with the aim to induce toxicity but not death or severe suffering*" (OECD 1998). Higher doses have already been chosen for other toxicity studies with whole GM soybean meal (Hammond et al. 1996; Appenzeller et al. 2008; Delaney et al. 2008). In addition to the presented data, information is lacking for the evaluation of any potential long-term (chronic) effects caused by unintended changes in the GM soybean that remained undetected.

5.3.4 Risks associated with pleiotropic effects

Occurrence of unintended and pleiotropic effects in event MON87769 were evaluated by different tests that were conducted under request of the different risk assessment agencies. These consisted of flanking sequence analysis, the comparative assessment (as described in Chapter 5.3.2), and toxicity studies with animal models (acute toxicity studies with the newly expressed proteins, 90-day feeding trial in rats).

With regard to the other information submitted by the applicant (bioinformatics analyses, no reported adverse effects in connection with the consumption of other desaturases, literature data, etc.) it was concluded by

EFSA (2014b) that there are no reasons to suppose that these specific desaturases would introduce safety concerns.

From the authors' point of view, uncertainties with respect to potential adverse effects associated with the consumption of the PjΔ6D and NcΔ15D proteins, which are produced in soybean MON87769 and do not have a history of safe use as part of human diet, result from the absence of repeated-dose studies (e.g. 28-day toxicity studies) with these proteins.

Furthermore, and as mentioned above, an evaluation of protein patterns through proteomics should be included in the risk assessment of GM plants with changed ω-3 fatty acid profiles, especially because of the involvement of proteins in all plant metabolic processes.

5.3.5 Exposure assessment

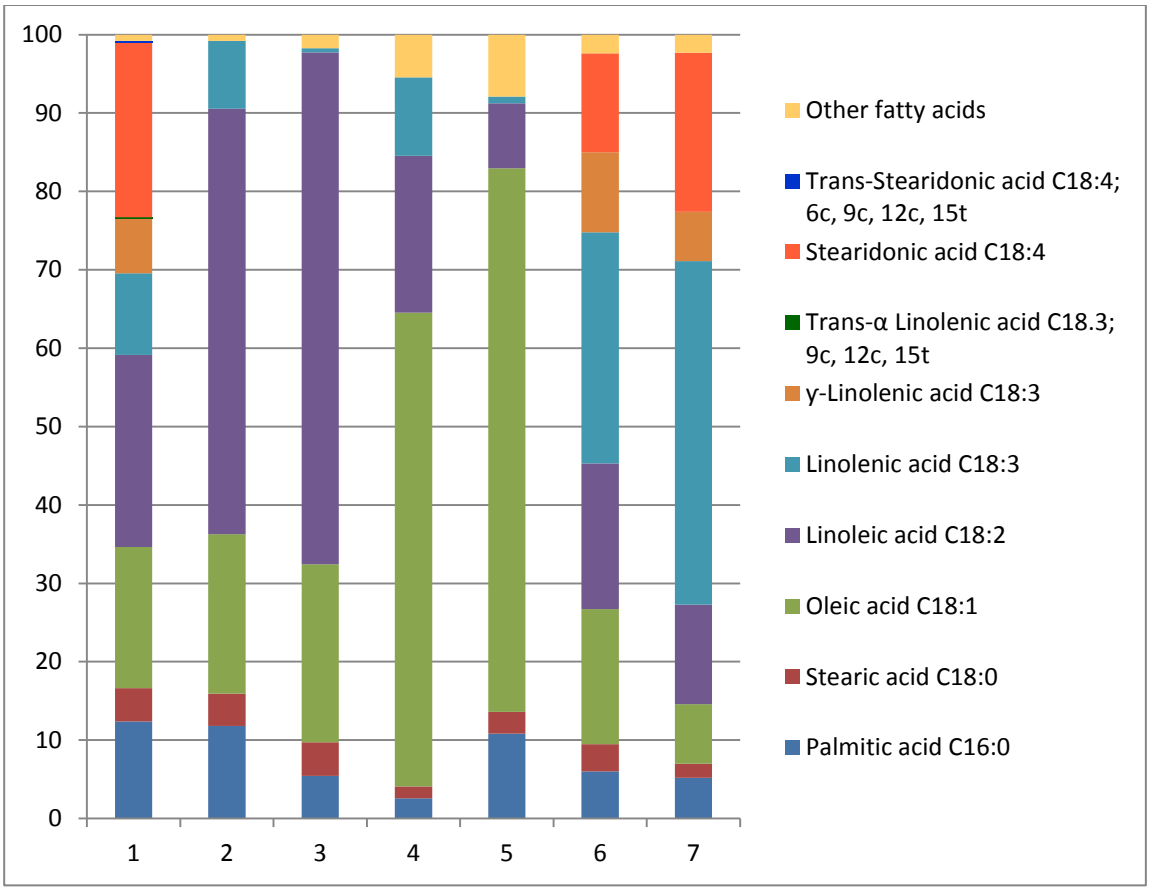
The second generation GMO soybean with enriched stearidonic acid has a significant alteration in its fatty acid profile. Following exposure assessment for this trait is focussed on the increased concentration of the unsaturated fatty acid stearidonic acid (SDA) and the new expressed by-products, which are trans-fatty acids.

Figure 2 demonstrates the differences of fatty acid oil content of soybean MON87769 oil compared to (non-GM) conventional vegetable (soybean-, sunflower-, rapeseed- and olive-) oils. The fatty acid profile of the novel foods echium oil and oil of *Buglossoides arvensis* is as well inserted, because they are comparable with regard of the stearidonic acid content to the GM soybean oil. As already described in chapter 5.2.5 the nutritional values of sunflower-, rapeseed- and olive oil derive from the nutritional.software nut.s which is based on the "Bundeslebensmittelschlüssel" BLS (Federal Foodstuffs Key).

The GMO soybean oil compared to the conventional soybean oil contains four additional fatty acids. These are stearidonic acid - which is the intended one, y-linolenic acid, *trans* stearidonic and *trans* α-linolenic acid. It should be pointed out that for *trans* fatty acids the intake should be as low as possible, according to the EFSA Panel on Dietetic Products, Nutrition, and Allergies (NDA) (EFSA 2010d). It was observed that the human body does not synthesise *trans* fatty acids (TFA). The sources for *trans* fatty acids are industrial foods which undergo a hydrogenation process and "naturally" from ruminants (Wagner et al. 2008).

Usually, native soybean oil does not contain *trans* fatty acids. Therefore, an exposure assessment is carried out for the intake of *trans* fatty acids of GM soybean oil.

Figure 9: Fatty acid profile (% of total fatty acids; mean) in GMO soybean oil compared to a variety of non-GMO conventional vegetable oils



1 = mean GMO soybean in g/ 100 g oil[1]
2 = mean non GMO soybean in g/ 100 g oil[1]
3 = sunflower in g/ 100 g oil[2]
4 = rapeseed in g/ 100 g oil[2]
5 = olive in g/ 100g oil[2]
6 = refined echium in g/ 100 g oil[3]
7 = *Buglossoides arvensis* in g/ 100 g oil[3]
[1] EFSA 2014b; [2] Bundeslebensmittelschlüssel (Federal Foodstuffs Key); [3]Technology Crops International 2013

The GM soybean expresses a high amount of stearidonic acid (SDA), and two *trans* fatty acids as by-products.

The Scientific Opinion of EFSA on Dietary Reference Values for fats in 2010 describes that *trans* fatty acids, both from ruminant sources and from industrial sources have adverse effects on blood lipids and lipoproteins. Prospective cohort studies demonstrate a consistent relationship between high intakes of *trans* fatty acids and increased risk of coronary heart disease (EFSA 2010d).

For estimating the *trans* fatty acids intake of the Austrian population, the food consumption database of the "Austrian Nutrition Report 2008" was used (Elmadfa et al. 2009). The Department of Nutritional Sciences collected data of a part of the Austrian population. Adults were interviewed with a 24-hour recall and the data of children was determined by a three-day dietary record.

Table 6 demonstrates a maximum intake scenario of the intake of *trans* fatty acids. It was assumed that the total consumption of the food categories "fats, oils and butter" were replaced by GM soybean oil. The dietary exposure assessment was performed by combining the mean concentration of the *trans* fatty acids of the GM soybean oil with the food consumption data of men, women and children. The mean and 95th percentile of the collective of the Austrian population was used.

Table 6: Maximum intake scenario of the intake of *trans* fatty acids from GMO soybean oil from men, women and children

Maximum	Men		Women		Children	
100% GMO	Collective		Collective		Collective	
	Mean	P95	Mean	P95	Mean	P95
fats, oils and butter (g/d)	35.0	72.6	32.3	66.7	18.9	35.5
sum of *trans* fatty acids (g/d)	0.13	0.26	0.12	0.24	0.07	0.13

P95 = 95th Percentile

The intake of *trans* fatty acids is very low and does not exceed the D-A-CH Reference values of *trans* fatty acids (see Table 7).

No exposure assessment of the minimum intake is demonstrated because already the intake in the maximum intake scenario is so low, that the other scenario is redundant.

The DGE (German Society of Nutrition) recommends keeping the daily intake of *trans* fatty acids as low as possible. The D-A-CH (German-Austria-Swiss Society of Nutrition) reference value of *trans* fatty acid intake is defined as lower than 1% of energy intake (DGE 2007).

The energy content of fats is 9.3 kcal. Thus, according to the recommendations of D-A-CH, men with an energy intake of 2400 kcal per day should not consume more than 2.6 g *trans* fatty acids per day (2400 : 100 : 9.3=2.6; see more information in Table 7).

Table 7: D-A-CH Reference values of *trans* fatty acids (DGE 2007; D-A-CH Referenzwerte 2012)

Age	Energy Intake		*Trans* fatty acids	
	m	f	m	f
25 - ≤ 51 years	2500 kcal/d[1]	2000 kcal/d[1]	2.7 g	2.2 g
7 - ≤ 10 years	1900 kcal/d[2]	1700 kcal/d[2]	2.04 g	1.8 g
10 - ≤ 13 years	2300 kcal/d[2]	2000 kcal/d[2]	2.5 g	2.2 g
13 - ≤ 15 years	2700 kcal/d[2]	2200 kcal/d[2]	2.9 g	2.4 g

[1] PAL = Physical Activity Level, PAL 1.4 = activity level for sedentary work and very low or none activity in the spare time (e.g. office worker, precision mechanic,…) is included
[2] no PAL included
m = masculine; f = feminine

The consumption of *trans* fatty acids from the GM soybean oil in the maximum intake scenario based on the food consumption of the collective (mean and 95th percentile) is less than 1% of the total energy intake and hence it is not a problem and acceptable.

EFSA states in its scientific opinion (EFSA 2010d), "*Trans fatty acids are not synthesised by the human body and are not required in the diet. Therefore, no population reference intake, average requirement, or adequate intake is set.*"

Based on the national *trans* fatty acid Regulation of the Republic of Austria, it is forbidden to produce or to place food on the market with a *trans* fatty acid content of more than 2 g/100 g in total fat (BGBl. II Nr. 267/2009). The GM soybean oil contains 0.36 g/100 g *trans* fatty acid and fits, therefore, with the *trans* fatty acid Regulation of Austria, because the *trans* fatty acid amount is negligible.

No adverse effects are expected of these *trans* fatty acids when conventional vegetable oils are replaced by MON87769 soybean oil (EFSA 2014b). Therefore, the authors follow EFSA's conclusion that no adverse effects

are expected of these *trans fatty* acids when conventional vegetable oils are replaced by MON87769 soybean oil.

Stearidonic acid (SDA) is a long chain polyunsaturated fatty acid (PUFA) and is a metabolic intermediate in the conversion of α-linolenic acid (ALA) to eicosapentaenoic acid (EPA) and docosahexanoic acid (DHA). SDA seems to be more readily metabolised than ALA to EPA in humans, with a conversion efficiency between 3:1 and 6:1 (SDA:EPA). An intake of around 750 mg SDA/day could theoretically have the same effect as an intake of 125-250 mg EPA/day (EFSA 2014b).

According to the applicant of soybean oil MON87769, the oil is foreseen to be added to foods as an ingredient that provides a precursor for EPA and DHA, in most cases replacing a portion of other oils in the diet. The SDA soybean oil is not intended to be used as oil for home uses. It will be used only by the food industry for convenience foods. Because of the high content of PUFAs it is unsuitable for high temperature operations such as frying (EFSA 2014b).

The applicant performed an exposure assessment of the SDA intake of adults (19 - 64 years) based on United Kingdom (UK) National Diet and Nutrition Survey and the US FDA information on serving sizes. Because of the lack of consumption data, the applicant could not provide a similar estimation for young children.

For the exposure assessment on SDA intake due GM soybean oil for the Austrian population, it is considered that the oil contains 22.35% SDA as demonstrated in the EFSA scientific opinion (EFSA 2014b). The food consumption is based on the data of the "Austrian Nutrition Report 2008" and refers to the food category "fats, oils and butter", which consists of butter, margarine, mayonnaise and vegetable oils. The target groups are the collective of men and women (19 - 65 years) and children (6 - 15 years). For the calculation, the mean and 95th percentile is used, and it is as well calculated on body weight (bw) basis (see Table 10 and Table 11). The operating figures of the bw for men, women and children are 81.5 kg, 63.6 kg and 39.7 kg, respectively (Elmadfa et al. 2009).

Table 8 demonstrates the average total intake per capita of SDA soybean oil for the Austrian population under the assumption that all fats, oils and butter would be substituted by MON87769 oil.

Table 8: Maximum intake scenario of the intake of SDA derived from GM soybean oil in men, women and children

Maximum	Men		Women		Children	
100% GMO	Collective		Collective		Collective	
	Mean	P95	Mean	P95	Mean	P95
fats, oils and butter (g/d)	35.0	72.6	32.3	66.7	18.9	35.5
SDA (g/d)	7.82	16.23	7.22	14.91	4.22	7.93

P95 = 95th Percentile; bw = body weight

Table 9 shows the scenario of a minimum intake of SDA per capita of men, women and children in Austria. For this exposure assessment it is estimated that 0.9% of the used soybean oil is genetically modified due to unavoidable contamination (see explanations in Chapter 5.1 "Method used for the exposure assessment").

Table 9: Minimum intake of stearidonic acid (SDA) derived from GM soybean oil in men, women and children

Minimum	Men		Women		Children	
0.9% GMO	Collective		Collective		Collective	
	Mean	P95	Mean	P95	Mean	P95
fats, oils and butter (g/d)	35.0	72.6	32.3	66.7	18.9	35.5
SDA (g/d)	0.07	0.15	0.06	0.13	0.04	0.07

P95 = 95th Percentile; bw = body weight

For comparison, the baseline intake of SDA from seafood in the United Kingdom is estimated to be around 20 mg/day (EFSA 2014b).

Table 10 illustrates a maximum intake scenario of the intake of SDA on a body weight basis.

Table 10: Maximum intake scenario of the intake of SDA derived from GM soybean oil in men, women and children (in g per kg bw/day)

Maximum	Men		Women		Children	
100% GMO	Collective		Collective		Collective	
	Mean	P95	Mean	P95	Mean	P95
fats, oils and butter (g/d)	35.0	72.6	32.3	66.7	18.9	35.5
SDA (in g per kg bw/d)	0.10	0.20	0.11	0.23	0.11	0.20

P95 = 95th Percentile; bw = body weight

Table 11 illustrates the minimum intake of SDA due GM soybean oil based on a body weight basis.

Table 11: Minimum intake of SDA derived from soybean oil in men, women and children (in g per kg bw /day)

Minimum	Men		Women		Children	
0.9% GMO	Collective		Collective		Collective	
	Mean	P95	Mean	P95	Mean	P95
fats, oils and butter (g/d)	35.0	72.6	32.3	66.7	18.9	35.5
SDA (in g per kg bw/d)	0.0009	0.0018	0.0010	0.0021	0.0010	0.0018

P95 = 95th Percentile; bw = body weight

The applicant also used the UK National Diet and Nutrition Survey to estimate the impact of replacing presently used vegetable oils in food with SDA-rich soybean oil on the intake of other fatty acids. Based on these data the dietary intake of n-3 PUFAs would increase by 2.70 - 2.85 g/day, whereas the intake of n-6 PUFAs would decrease by 0.85 - 0.62 g/day. The total saturated fatty acid intake would increase by 0.54 - 0.79 g/day (EFSA 2014b).

It is necessary to point out that the oil of GM soybean oil MON87769 is comparable to the fatty acid profile of refined echium oil and *Buglossoides arvensis* oil which are classified as novel foods due to the circumstance they were not consumed in high amounts before the 15th of May 1997. All of these oils contain high amounts of ω-3-fatty acids, which are healthy ingredients regulated under the Health Claim Regulation (EU) No 432/2012 (EC 2012; EFSA 2010e; EFSA 2011d). Otherwise, according to Regulation (EU) No 432/2012, it must be ensured that there is no overconsumption of ω-3-fatty acids. The consumer should be informed that there is, for example, a positive effect when 3 g DHA and EPA (per day) are consumed in combination but that an intake of 5 g DHA and EPA must not be exceeded (EC 2012).

The applicant could not demonstrate an exposure assessment of children, who are of most interest. EFSA recommends performing a post-market monitoring plan for the exposure assessment if a product is placed on

the market (EFSA 2014b). If a novel food is placed on the market, the applicant has also to provide detailed information with regard to the specifications of the product and the intended uses. Therefore, the applicant has to declare in what food categories the food ingredient will be used.

In addition, the labelling, the correct usage (like in this case "no frying") and the dietary intake has to be stated. It should also be taken into account that there are other ω-3 fatty acid sources like fish, fish oil (used in food supplements) and a lot of novel food products (e.g. oil from microalgae, krill oil) on the market.

5.4 GM plants with a very low content of coeliac-toxic epitopes (gliadin)

The design of a GM wheat plant with a very low content of coeliac-toxic epitopes aims at inhibition of natural gliadin and production of modified glutenin polypeptides, so that food material prepared from those plants (e.g. flour, dough, bread) can be consumed by persons suffering from coeliac disease. Coeliac disease is a T-cell-mediated food allergic disorder, it is non-IgE-mediated (Kupper 2011). It is triggered by ingestion of gluten, which is a protein fraction contained in the cereals wheat, rye, and barley, in genetically susceptible persons (WAO 2004; Catassi et al. 2007).

It was early recognised that coeliac disease was caused by gliadin proteins present in wheat. It is currently known that persons who suffer from coeliac disease are also negatively affected by some wheat glutenin proteins and proteins present in rye and barley (Vader et al. 2002; Catassi et al. 2007). Gliadin and glutenine proteins together represent the main protein fraction in wheat. The gliadins belong to the wheat prolamine and the glutenines to the wheat gluteline fraction. Rye and barley do not contain gliadins but are members of the same tribe (Triticeae) as wheat. Because of the close relationship of the species of this tribe, the prolamine proteins of rye and barley (named secaline and hordeine) contain polypeptides with sequences that are homologous to wheat gliadins (Wieser 2001).

There is evidence for an association of coeliac disease to prolamines with high ratios of the amino acids glutamic acid (E), glutamine (Q) and proline (P). Potential toxic amino sequences were identified to contain the amino acid motifs PSQQ and QQQP, which occur frequently in wheat, rye and barley prolamines. Other sequences of gluten peptides can also stimulate coeliac disease. Patients suffering from coeliac disease, thus, usually are also negatively affected by proteins from rye, barley and oats. In addition, a number of humans suffer from general intolerance to glutens which is not T-cell-mediated. The only cure for patients is a life-long gluten-free diet strictly avoiding all food and pharmaceutical compositions containing wheat, rye and barley (Wieser 2001; Catassi et al. 2007).

The concept of the development of a GM plant with a very low content of coeliac-toxic epitopes obtained by RNA interference was patented as new invention a few years ago in the United States and is described by a German research team (Becker et al. 2009; Becker et al. 2012):

The gliadins mostly impart extensibility to a dough whereas glutenins impart both elasticity and extensibility. The large glutenin molecules contribute less to extensibility, but the smaller glutenin molecules have the reverse effect. This means that the molecular weight distribution of the glutenin monomers as well as the ratio of glutenin to gliadin highly influences the visco-elasticity of the dough. Gluten proteins are important in food processing and are to be found in dough (bread, biscuits, etc.), coating on confectionery, and also coating on pharmaceutical tablets.

Gliadins and low molecular weight (LMW) glutenins contain 6 or 8 cysteine residues that form homologous intramolecular disulfide bonds. Two of the cysteine residues present in LMW glutenins form intermolecular disulfide bonds that are responsible for the aggregative nature of LMW glutenin subunits.

The design of a GM plant with a very low content of coeliac-toxic epitopes aims at encoding modified glutenin polypeptides, wherein the sequences encoding one or more cysteine residues responsible for intermolecular crosslinking were substituted by sequences encoding other amino acids. Also part of the invention is that expression of all endogenous gliadin genes in the genome of the plant is inhibited by RNAi interference.

In the transformation event T0, all genes encoding a gliadin polypeptide are inactivated, and therefore this method provides cereal plants having seed wherein no natural gliadin polypeptides are expressed. At the same time the modified glutenin which does not contain any cysteine residue responsible for intermolecular crosslinking is expressed as a gliadin substitute (Becker et al. 2009).

5.4.1 Molecular characterisation

The molecular characterisation of transgenic wheat varieties with a reduced α-gliadin content obtained by RNA interference is described as presented in Becker et al. (2012).

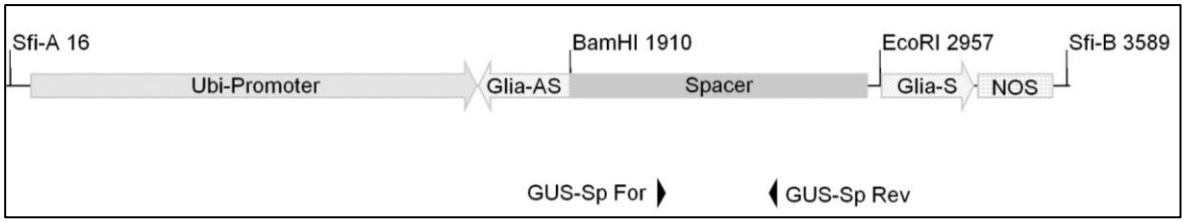
5.4.1.1 Construction and transformation of the transgenic insert

The α-gliadin genes in wheat were silenced by an RNAi approach:

Two 313 bp fragments (Glia-AS and Glia-S) from the 5' coding region of an α-gliadin sequence showing approx. 90 - 100% homology to 29 α-gliadin mRNA and genomic sequences deposited in Genbank® (NCBI 2014) were cloned in antisense direction into the pGliaRNAiSpacer vector as depicted in Figure 10.

Upon transcription from the ubiquitin promoter from maize a "hairpin" (hpRNAi) is formed containing a spacer loop of 1002 nts, which originated from the ß-glucuronidase gene (uidA/gus). This artificial hairpin structure activates RNAi induced gene silencing in plants which leads to a decreased expression of the targeted gliadin proteins.

Figure 10: Schematic representation of the pGliaRNAiSpacer vector (Becker et al. 2012)



The pGliaRNAiSpacer vector construct was transferred into the hexaploid winter wheat Triticum aestivum cv. Florida by biolistic transformation and co-transformed with the vector pCaIneo containing the antibiotic resistance marker gene nptII for the selection of transgenic calli under exposure to kanamycin. 111 independent transgenic wheat lines could be regenerated. The integration of the pGliaRNAiSpacer vector was confirmed by Southern blot analysis using a DIG-labeled uidA(gus) gene probe. The number of integrated gene copies determined in BamHI Southern blots (cutting once in the construct; see Figure 10) was between one and up to 20. The co-transformation frequency was 64%. All stably transformed lines were further analysed.

5.4.1.2 Expression and stability of the pGliaRNAiSpacer expression cassette in transgenic wheat

To check the functionality of the transgenic insert RNA from wheat leaves was isolated and analysed by insert specific RT-PCR. In seven out of nine T0 transgenic lines the expected amplification product of 396 bp could be detected. Specificity of the RT-PCR signal was verified by Southern blots. All primary transformants were self-

fertile and the seed set and seed morphology was comparable to wild type plants. The stable integration and expression of the RNAi construct and the transmission of the phenotype were successful determined in the following generation in self-pollinated plants.

5.4.1.3 Gene expression profiles

To check whether the transferred pGliaRNAiSpacer cassette fulfilled its aim (RNAi induced silencing of α-gliadin) and to detect potential unintended effects due to the genetic modification the protein composition of the transgenic wheat was determined by RP-HPLC and isoelectric focussing/two-dimensional gel electrophoresis.

The total protein content of the transgenic wheat lines appeared to be unchanged compared to the wild type plants (the RP-HPLC profiles of the albumin/globulin fraction from the wild type and transgenic lines were almost identical). In contrast, the RP-HPLC profiles of gliadins were in some transformants substantially different. In all cases the profiles of ω-gliadins and γ-gliadins were unchanged, whereas those of α-gliadins varied remarkably. Some lines did not show any difference to the wild type. In several other lines a strong reduction of α-gliadins was observable. However, the loss of α-gliadins was compensated by the increase of other storage protein types.

The determination of the relative amounts of the total soluble gliadin fraction and α-gliadins allowed the establishment of four transgenic groups (technically co-eluted during RP-HPLC with ethanol soluble glutenins):

1) Seven transgenic wheat lines contained 30 - 36% α-gliadins.
2) Seventeen lines contained 24 - 30% α-gliadins.
3) Forty three lines contained 18 - 24% α-gliadins.
4) Twenty four lines contained 12 - 18% α-gliadins.

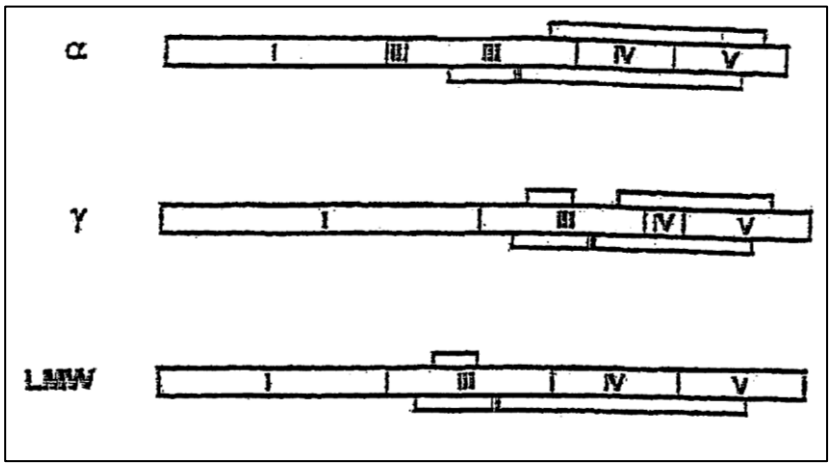
N-terminal amino acid sequence analysis revealed that no α-gliadins were present in a single line of group 4. This was interpreted that the applied RNAi technique allowed eliminating α-gliadins completely.

The alcohol insoluble glutenin fraction was unchanged (compared to the wild type) in Group 1. However, low molecular weight glutenins were reduced or missing in Groups 2-4, which might be an indication for off-target effects of the RNAi modification. In spite of the altered protein composition of the transgenic wheat the dough and bread making properties were comparable to those of the wild type showing that α-gliadins were not an essential protein group for baking quality.

5.4.1.4 Introduction of dual alterations into the wheat genome: US Patent No. 7501558

In US Patent No. 7501558 (Becker et al. 2009), the silencing of one or all gliadin genes by RNAi or antisense technology (as exemplified in Section 5.4.1.1) is combined with an alteration of a low molecular weight glutenin gene. The transgenic plant contains - in addition to the RNAi silencing constructs - a LMW glutenin with no cysteine residues responsible for intermolecular crosslinking via disulfide bridges. This modified LMW glutenin is expressed as a substitute for the missing gliadin proteins and allows retaining the baking characteristics of wheat flour by concomitantly significantly reducing the allergic reactions of sensitive consumers. In general the applied glutenin genes should show low (< 50%, preferably < 30%) of homology in the 5’ region of similar gliadin sequences (for comparison see Figure 11). The applicant describes the process using a modified form of the LMWa3 glutenin gene where the codons for two cysteines have been replaced by serine.

Figure 11: Schematic comparison of α- and γ-gliadin and LMW glutenin sequence domains (Becker et al. 2009)



The RNAi expression cassette and the expression cassette containing the modified LMW glutenin are preferentially co-transferred via biolistic transformation scutelli from 14 day old zygotic wheat embryos. However, *Agrobacterium*-mediated transformation is also covered by the patent application. Selection of transformants is facilitated via kanamycin and 2,4-D.

Successful transformation events and insert copy numbers are verified by insert-specific probes via Southern blots. The expression of the modified glutenin gene (*LMWa3.DELTA.2C*) is determined by SDS-PAGE and western blots.

5.4.2 Comparative assessment

The comparative assessment aims to identify differences in composition, agronomic performance and phenotypic characteristics between the GM plant with a very low content of coeliac-toxic epitopes and derived food and feed (e.g. bread) and its comparator. The comparison depends on the availability of a conventional comparator, which is defined as a non-GM genotype with a genetic background as close as possible to the GM plant (EFSA 2011; EC 2013a).

Adequate comparators can be either non-GM lines with a genetic background as close as possible to the GM plant under assessment in case of sexually propagated crops, or isogenic varieties in case of vegetatively propagated crops. Wheat, barley or rye are sexually propagated crops, and therefore non-GM lines with a genetic background as close as possible to the GM plant are required.

EFSA (2011b) points out that the extent to which these non-GM comparators are genetically related to the GM plant depends on the breeding scheme used for the production of the GM plant, and also that, if no conventional comparator is available, the risk assessment has to be carried out as for other novel foods. In principle, where significant compositional changes have been targeted, appropriate comparators are not available, and in such cases a comprehensive safety/nutritional assessment on the GM plant per se needs to be conducted. Such a risk assessment will usually consist of information and evaluation of:

- the characteristics of the donor organisms and recipient plant,
- the genetic modification and its functional consequences,
- agronomic and phenotypic characteristics of the GM plant,
- compositional characteristics of GM plants and derived food and feed,
- the potential toxicity and allergenicity of gene products (proteins, metabolites) and the plant and its derived products,
- the dietary intake and potential for nutritional impact, and

- the influence of processing and storage on the characteristics of the derived products.

The patent document indicates that the development of a GM plant with a very low content of coeliac-toxic epitopes may result in significant compositional changes. The production of gliadins, which account for approximately 33% of total protein in wheat (Wieser 2001), will be inhibited, and instead of the gliadins modified glutenines not forming intermolecular disulfid bridges will be encoded by the transgenes inserted (Becker et al. 2009). And so, as the gluten fraction is only modified, the compositional analysis, besides a comparison of the OECD endpoints, could focus on the evaluation of significant differences in gluten fractions and newly expressed proteins, the modified LMW glutenins. Especially, the concept behind these GM plant is that the gliadin proteins are suppressed by RNAi technology resulting in a food or feed prepared from the GM wheat which is gliadin-free.

From the current knowledge (insufficient experimental data on the GM plant event), it is highly unclear whether the GM plant (transformation event) has to be classified as being substantially different to its parental (non-GM) lines, and whether the concept of substantial equivalence may be applicable.

In case the concept of substantial equivalence is applicable, which has to be decided on the basis of information on the modifications of the genetic endogenous composition, metabolism and physiology of the plant (ADAS 2013), the endpoints are to be selected in accordance with appropriate OECD documents (EFSA 2011a). For GM wheat with a very low content of coeliac-toxic epitopes the endpoints for compositional analysis would be taken from the respective OECD consensus document for new varieties of bread wheat (OECD 2003):

Usually the endpoints considered include proximates (including moisture and total ash), key macro- and micro-nutrients, anti-nutritional compounds, natural toxins, and potential allergens. For wheat kernels the following endpoints are mandatory: carbohydrates, proteins, vitamins (thiamine, riboflavin, niacin, pyridoxine, folic acid), minerals, lipids, and other components which include phenolic acids, lignans and flavonoids.

The phenolic acids consist of ferulic acid, vanillic, p-coumaric, protocatechuic, syringic, p-hydroxybenzoic, caffeic, and genitisic acids. Ferulic acid is ester-linked to specific polysaccharides (the arabinoxylans), which form 65% of the aleurone cell walls. Bacterial enzymes in the human colon slowly and partially degrade the aleurone cell walls. This degradation results in the release of feruloylated oligosaccharides, which can then be further degraded to release ferulic acid. Ferulic acid is an antioxidant.

Flavonoids are phenolic compounds that occur widely in plants and have antioxidant properties. The highest concentration of flavonoids is in the wheat germ followed by the wheat bran. Wheat bran contains small amounts of the flavonol catechin and proanthocyanidin, which are polymers based on flavonol units. The lignans are phenolic dimers and mainly present in the wheat bran. They are converted in the large intestine to mammalian lignans.

The comparison also should consider other cereal compounds as dehydrated distillers' grains (DDG), which are a by-product of the fermentation of cereal grains to make ethanol for industrial uses and particularly contain high concentrated protein. Manufacturers usually dry distillers’ grains to yield DDG and sell them as an ingredient for animal feed. However, it should be noted that wheat is generally only an economical choice as a fermentation substrate if the distillers' grains are sold as a human food. It is known that DDG, including those from wheat, have been used as ingredients in baked goods and other foods to enhance protein and dietary fibre content (OECD 2003).

Additionally, the following anti-nutrients that occur in cereals (OECD 2003) need to be part of an appropriate comparative assessment study:

i) protease inhibitors (may decrease the digestibility and biological value of ingested protein)

ii) amylase inhibitors (may affect the digestibility of starch)

iii) lectins (bind to certain carbohydrate groups on cell surfaces, such as intestinal epithelial cells, where they can cause lesions and severe disruption and abnormal development of the microvilli)

iv) phytic acid (chelates minerals such as iron, zinc, phosphate, calcium, potassium and magnesium. The bioavailability of trace elements such as zinc and iron can, thus, be reduced by the presence of phytic acid in monogastric animals, although in humans, phytic acid does not seem to have a major affect on potassium)

The OECD (2003) also notes and it should be taken into account for the risk assessment that gliadin has been reported to be an allergen prominently involved in cases of non-exercise-induced anaphylaxis in young children resulting from the ingestion of wheat flour. As said before, and particularly relevant for the invention, wheat, along with other gluten-containing cereals such as rye and barley, is associated with coeliac disease, which affects genetically predisposed individuals (OECD 2003).

The comparative assessment should take into consideration any of the abovementioned points. It is, furthermore, clear that a thorough comparison of proteins and polypeptides present in the prolamine and glutenine fractions (gliadins, gluteline, etc.) between the GM plant and its conventional comparator is an absolute requirement.

In case no comparative assessment can be implemented a comprehensive safety/nutritional assessment on the GM wheat has to be conducted. Such a risk assessment strategy has to be based on the four obligatory steps in risk assessment: hazard identification, hazard characterisation, exposure assessment and risk characterisation.

Regulation (EU) No 503/2013 states, "*Where no appropriate conventional counterpart can be identified, a comparative safety assessment cannot be made and consequently a safety and nutritional assessment of the genetically modified food or feed shall be carried out as for novel foods falling within the scope of Regulation (EC) No 258/97 of the European Parliament and of the Council that do not have conventional counterparts*" (EC 2013a). Such a thorough risk assessment obviously would include different kinds of animal testing. This would be the case, in particular if the modification affects several metabolic pathways of the plant.

5.4.3 Toxicological and allergological assessment

Recent developments in reducing gluten components relate to transgenic wheat comprising a modified low molecular weight glutenin. They also concern using seed and products thereof and which are derived from in the preparation of food and/or feed which is primarily gliadin-free (Becker et al. 2009). Being in principle beneficial for patients suffering from coeliac disease, with reduction or removal of gliadin only, nevertheless some safety points have to be taken into consideration:

More recent classification, according to primary amino acid structure, reveals not only great heterogeneity but also similarities between different gliadin and glutenin proteins. Peptides derived from both groups are immunostimulatory in coeliac disease, and it is highly probable, therefore, that glutenin proteins are toxic (Howdle 2006). Thus, reduction or non-expression of gliadin alone does not completely eliminate the health problems of coeliac patients.

Different diseases and intolerances are associated with the consumption of wheat. Wheat allergy is a reaction to proteins found in wheat, triggered by the immune system. Coeliac disease is a condition in which the lining of the small intestine is damaged by gluten. Non-coeliac gluten sensitivity (gluten intolerance) is when symptoms similar to coeliac disease are experienced, but there are no associated antibodies and no damage to the lining of the gut. The exact role of the immune system in non-coeliac gluten sensitivity is unclear and further research is needed.

The definition of non-coeliac gluten sensitivity goes back to 1986, but only recent advances enable to make a clear differentiation between coeliac disease and gluten sensitivity. So there is now growing evidence that, besides those with coeliac disease or wheat allergy, there are patients with gluten sensitivity in whom neither allergic nor autoimmune mechanisms can be identified.

It has been estimated that for every person with coeliac disease there should be at least six or seven people with non-coeliac gluten sensitivity. Gluten sensitivity may, therefore, affect 6-10% of the general population. The observed clinical symptoms can overlap with those of coeliac disease, irritable bowel syndrome, and wheat allergy; and patients show a resolution of symptoms when started on a gluten-free diet. Currently there are no laboratory biomarkers specific for gluten sensitivity, and the diagnosis is based on exclusion criteria; elimination of gluten-containing foods from the diet followed by an open challenge is most often used to establish whether health improves with the elimination or reduction of gluten from the patient's diet (Rostami and Hogg-Kollars 2012). With reduction or absence of gliadin for people with gluten sensitivity their problems are not solved but continue to exist.

In the European Union, a maximum level of 20 mg/kg gluten is allowed for products declared as "gluten-free", and 100 mg/kg gluten for products declared as "very low gluten" respectively (EC 2009). These values are in principle based on an EFSA Scientific Panel Opinion (EFSA 2004). With only gliadin reduction or removal the compliance with such threshold levels is not guaranteed.

Rather recent developments, for instance, patented by United States Patent 7501558 state that the gliadin-free foodstuff derived from transgenic wheat is beneficial for patients suffering from coeliac disease and/or other forms of gluten intolerance. Besides the reduction or absence of gliadin, the patent claims a modified glutenin polypeptide to be introduced as a gliadin substitute, and that for the purpose of preparing foodstuffs from the seed of the transgenic wheat the modified glutenin polypeptide serves as a gliadin substitute (Becker et al. 2009).

But the modified glutenin may represent an allergological risk by itself. Therefore, it should be subjected to a stepwise procedure within a decision tree for assessment of the allergenic potential of foods derived from genetically engineered food crops (Metcalfe et al. 1996).

In addition to amino acid sequence homology comparison, specific serum screening, and pepsin resistance and *in vitro* digestibility tests, the EFSA Guidance for risk assessment of food and feed from GM plants (EFSA 2011a) recommends, "*Although additional tests including in vitro cell based assays or in vivo tests on animal models have not been validated so far for regulatory purposes, they may be considered useful to provide additional information e.g. on the potential of the newly expressed protein for de novo sensitisation.*"

To assess the potential for immunological sensitisation, tests of interest are the Guinea Pig Maximisation Test or the Local Lymph Node Assay, according to the OECD Guideline No. 429 (OECD 2010). Other methods, such as the Mouse Intra-Nasal Test and the Brown Norway rat model could be considered as complementary information (Van Haver et al. 2003). Should tests turn out to be positive, then there would be no advantage at least for gluten sensitive individuals, but a continuing health hazard, leastwise for a specific population.

Additionally, potential unintended effects may lead to potential long-term (chronic), reproductive or developmental effects, and therefore appropriate toxicity studies need to be considered.

5.4.4 Risks associated with pleiotropic effects

Unintended effects (e.g. pleiotropic effects) can be caused by transformation and random insertion and need, therefore, to be adequately addressed during the risk assessment of GM plants and derived food and feed. One step during risk assessment for identification of unintended effects is the comparison between the GM plant and its conventional comparator and the search for unintended differences (lack of equivalences), usually following the first step, the molecular characterisation of the transgenic insert and the flanking sequences.

Unintended effects are known to be associated with the site of transgene integration, potentially resulting in changed metabolism, novel fusion proteins, or other pleiotropic effects that could compromise product safety (Szwacka et al. 2012). As genetic engineering could produce novel fusion genes and novel fusion proteins in GM plants, an adequate molecular characterisation of the insertion site needs to be conducted identifying, amongst others, potential interruptions of known genes and novel open reading frames. Usually, for molecular characterisation bioinformatics analyses of putative ORFs are carried out. On the basis of EFSA requirements, additional studies (e.g. northern blot analysis) add to the risk assessment and are to be conducted, especially for silencing approaches or where biochemical pathways have been intentionally modified. Both techniques (silencing, modification of biochemical pathways) are employed for producing GM plants with a very low content of coeliac-toxic epitopes (Becker et al. 2009). Therefore, information to demonstrate whether the inserted/modified sequence results in intended changes at RNA or metabolite levels has to be collected during risk assessment (EFSA 2011a). Any aberrations from the intended changes can be seen as evidence for an unintended effect not fulfilling the original objective of the genetic modification.

The design of a GM plant with a very low content of coeliac-toxic epitopes, as depicted and described in the patent document, suggests a high degree of metabolic changes induced by genetic engineering (Becker et al. 2009):

i) The document refers to at least two transformation steps as of the production of the desired trait, "*The transgenic plant may further comprise a genetic alteration, which inhibits or reduces the expression of at least one endogenous gliadin gene. The genetic alteration may be introduced before, together with or after the transformation with the nucleic acid sequence as set forth in SEQ ID NO:1.*" (Alternatively, the use of a gliadin-null variety is suggested, but without further elucidation of the actual availability of such a wheat variety.)

ii) The necessity that several genes need to be targeted is noted, "*According to a preferred embodiment of the invention all genes encoding a gliadin polypeptide are inactivated in the plant genome.*"

iii) It is also remarked, "*Differences in the sequence homology of at least 75% are accepted, "In a preferred embodiment of the present invention, the used glutenin gene sequence comprises a sequence having a homology of at least 75% to the sequence shown in SEQ ID NO: 1. Preferably the sequence homology is at least 85% or at least 95% to the sequence shown in SEQ ID NO: 1.*"

An overview of the wheat endosperm protein population is presented by Vensel et al. (2005) who studied the developmental changes of wheat storage protein patterns. This study gives an insight into the complexity of the protein fraction in grain and also shows that remarkable variations exist for the different stages of grain development.

The different prolamine and gluteline in wheat comprise:

- α-gliadins
- γ-gliadins
- ω-gliadins
- high molecular weight glutenin
- low molecular weight glutenin (groups B, C and D)

The ω-gliadins do not form intramolecular disulfide bonds because of the absence of cysteine residues, whereas α- and γ-gliadins form intramolecular disulfide bonds. The polymeric glutenins can form intra- as well as intermolecular disulfide bonds. The glutenin polymers can reach up to several million Daltons. They release single polypeptides classified into high molecular weight glutenin (HMW) subunits and low molecular weight glutenin (LMW) subunits, ranging from 70 000 to 90 000, and from 20 000 to 45 000, respectively.

LMW glutenin subunits can be divided into the B, C, and D groups. The majority is present in the B group. The C group contains mainly glutenin polypeptides with α-type or γ-type gliadin sequences. Their incorporation in the polymeric fraction of the wheat gluten is likely due to mutations altering the number of cysteine residues. The D-group of LMW subunits consists of polypeptides with typical ω-gliadin sequences that are similar to the C-type LMW subunits. This means that their presence in the glutenin polymers is likely due to mutations resulting in the presence of at least a single cysteine residue (Scossa et al. 2008).

Important data were derived in another wheat genome experiment conducted to identify abundant flour proteins in a wheat cultivar. A quantitative two-dimensional electrophoresis study was carried out and 233 proteins were successfully identified which could be assigned to 157 wheat gene sequences. Interestingly, it was found that many genes for wheat flour proteins are not expressed.

Proteome maps would facilitate future studies addressing the effects of genetic and environmental factors on the development and quality of wheat grain. This experimental data, in any case, highlight the value of proteomics analysis for characterising biochemical processes in connection with wheat storage proteins (Vensel et al. 2005). For an evaluation of pleiotropic effects information on the transcripts and protein synthesis would be required.

As further proteome maps will be developed and the techniques for characterisation of GM plants using proteomic approaches will improve, the application of such techniques can be seen as an appropriate tool in the risk assessment of the transgenic wheat with a very low content of coeliac-toxic epitopes. Other omics techniques, e.g. transcriptomics measuring the mRNA abundance from a given tissue source, should be considered as it is highly unclear what pleiotropic changes are to be expected when wheat gluten metabolic pathways are targeted by genetic engineering leading to substantially modified GM plants.

Important data for evaluating unintended effects can also be derived from appropriate animal feeding studies. Regarding the value of such data on toxicological and allergological assessment of GM wheat with a very low content of coeliac-toxic epitopes, it is referred to the discussion provided in Chapter 5.4.3.

5.4.5 Exposure assessment

The exposure assessment is an obligatory step of GMO risk assessment and substantial for marketing of second generation GM plants.

Because of the genetic modification of ingredients and properties of plants a new product arises. In case of the transgenic wheat with a very low content of coeliac-toxic epitopes, the gliadin content is reduced. The intended use of this gliadin reduced wheat is feed and food such as dough, batters, pastries, cookies, pasta,

wafers, bread and confectionery. This GM crop is targeting people with coeliac disease and people who are suffering from permanent gluten intolerance. Wheat (durum, spelt, kamut), rye, barley and oats contain gluten and can cause adverse health effects to coeliac disease patients. Thus, they usually need to avoid those crops a lifelong.

The process of removing gluten from gluten-containing grains is difficult and low amounts of gluten may remain in the final products. Therefore, it is important that products with a different gluten concentration are properly labelled so that coeliac patients can choose food products appropriate for their needs. Based on Regulation (EC) No 41/2009 Article 3(1), it is obligatory that foodstuff for people intolerant to gluten, consisting of or containing one or more ingredients made from wheat, rye, barley, oats or their crossbred varieties, the gluten level should not exceed 100 mg/kg in the food as sold to the final consumer. Furthermore, the labelling "gluten-free" is only allowed if the gluten content does not exceed 20 mg/kg in the food as sold to the final consumer (EC 2009).

In case of the GM wheat with reduced gliadin content, it is important to discover any hazards. Even if the protein amount which causes intolerance is lowered it is not for sure that the GM wheat is either suitable or safe for coeliac people or people with gluten intolerance. Caused by the genetically modification of the wheat, new proteins replace the gliadins and may trigger unwanted effects that could harm human health.

A reduction of the gliadin concentration was targeted in two different studies on GM wheat. The first study aimed at reducing of the α-gliadin fraction, the other one at reducing of the γ-gliadins. Both studies together do not indicate that a total reduction of the gliadin fraction in wheat can be achieved (Becker et al. 2009; Becker et al. 2012). Based on the current information available sufficient quantitative data regarding a reduced total gluten content is lacking. Hence, no exposure assessment can be implemented.

If a GM wheat with a very low content of coeliac-toxic epitopes was to be placed on the market, it would be indispensable to risk assess any newly expressed proteins. They would have to be safe and not harm human health. The gluten concentration should not exceed 20 mg/kg in foodstuff to label it "gluten free" and an exposure assessment would have to be performed.

5.5 GM plants with enhanced vitamin (pro-vitamin) content

The enhancement of vitamins in plants by genetic engineering methods for preventing vitamin deficiencies has been pursued in many studies. Most of the GM plants with high vitamin content are modified by targeting the carotenoid biosynthesis of the plant organism, aiming at the enhancement of β-carotene. A prominent example is the β-carotene increased rice variety, well known as "Golden Rice", which has been developed to combat vitamin A deficiency mainly in Asia (Paine et al. 2005). Other vitamin biosynthesis pathways have also been targeted by genetic engineering (e.g. tocopherol) (Yusuf et al. 2010), although, β-carotene enhancement seems to be the most important vitamin GM plant trait at present.

The first three steps of the carotenoid biosynthesis pathway are mainly in the focus of present research in GM plant engineering to enhance the β-carotene content of the plant. It starts with the condensation of two molecules of geranylgeranyl diphosphate (GGPP) leading to phytoene, which is then converted to prolycopene and further desaturated to yield lycopene. Different plant enzymes take part in this process: The phytoene synthases PSY1 and PSY2, the phytoene desaturase PDS, the ζ-carotene desaturase ZDS, and the carotene isomerase CrtISO. Lycopene can be converted to either α-carotene or β-carotene. The latter is the starting point for the synthesis of xanthophyll carotenoids as zeaxanthin or violaxanthin (Diretto et al. 2010).

β-Carotene, which belongs to the group of provitamins A, is the most meaningful of all carotenoids, because it is an important dietary precursor of vitamin A in the human body (Shewmaker et al. 1999). Vitamin A, which

includes retinol, retinal and retinoic acid, has essential functions as it is required for normal growth and development, and the functioning of the retina. Vitamin A also influences cell differentiation and contributes to the antioxidant defence mechanisms of the body (Gerster 1997).

In some bacteria species (e.g. *Erwinia*), the production of β-carotene from GGPP is mediated only by three enzymes: phytoene synthase CrtB, phytoene desaturase/isomerase CrtI, and lycopene cyclase CrtY. The most important strategy leading to an enhancement of β-carotene in GM plants is the tissue-specific overexpression of these bacterial genes or similar biosynthetic genes in the plant genome (Diretto et al. 2010). So, in the Golden Rice events I and II, phytoene desaturase/isomerase CrtI is overexpressed in combination with an expression of a transgenic phytoene synthase PSY from either daffodil and or maize (Paine et al. 2005).

There are other examples for the genetic modification of the carotenoid biosynthesis using one of these transgenes (*crtB*, *crtI*, *crtY*):

GM tomatoes converting lycopene, which is the major storage carotenoid of the tomato fruit, into provitamin A were developed using *crtY* genes (lycopene β-cyclases from *E. herbicola* and from the higher plant daffodil). These transgenic tomato events showed a 50% increase in total carotenoid (Apel and Bock 2009).

An oilseed rape variety was genetically modified by using an expression cassette containing a bacterial phytoene synthase gene *crtB*. This transformation led to an increase of carotenoids in seeds, a tissue that does not normally accumulate them indicating the flexibility of the pathway and accumulation mechanism. The levels of carotenoids in the seeds were about 1500 µg/g fresh weight (fw), which is higher than many other plant sources like e.g. carrots (Shewmaker et al. 1999). Another Brassica species related research concerned the development of a high β-carotene variety of mustard with good agronomic qualities and specifications that could promote adoption by Indian farmers (Brink 2003).

An US patent from 1997 provides information that carotenoid accumulation using a biosynthetic phytoene synthase enzyme encoded by the *crtB* gene can be induced in plants as carrot, melon, squash, red guava, passion fruit, mango, red papaya, avocado, cherry, tangerine, mandarin, palm, cucumber, apricot, and peach (Hauptmann et al. 1997).

Vitamin A enrichment through gene technology in major staple crops like maize and potato has also been achieved. In maize, the carotenoid pathway was targeted by the overexpression of the bacterial genes *crtB* and *crtI* leading to an increase of the total carotenoid level of up to 34-fold with an accumulation of β-carotene in the endosperm (Aluru et al. 2008).

Different studies on the enhancement of β-carotene in potato tubers were performed using a variation of transgenic approaches related to the three enzymes CrtB, CrtI, CrtY. In an experiment by Ducreux et al. (2005), *crtB* was put under the control of the tuber-specific patatin promoter resulting in an increase of total carotenoids from 20 µg/g dry weight to 78 µg/g dry weight in the most affected transgenic line. Another study aimed at a tissue-specific overexpression of a mini-pathway containing all three bacterial genes (*crtB*, *crtI*, and *crtY*). In that approach, potato tubers containing up to 47 µg/g dry weight of β-carotene. With an > 3600-fold increase this β-carotene accumulation is said to be the highest vitamin modification reported for any of the major staple foods (wheat, maize, rice, potato) by genetic engineering (Diretto et al. 2007a).

The following evaluation of the risk assessment of GM plants with enhanced vitamin content is based on the experiments and evaluation of high β-carotene potato tubers of Diretto et al. (2007) and Diretto et al. (2010). These studies provide the most useful information on the underlying molecular mechanisms, and the

published experimental data allow valid conclusions on potential risks associated with genetically modified vitamin enhanced plants.

5.5.1 Molecular characterisation

The successful development of genetic constructs to induce higher provitamin A levels in major staple crops is exemplarily described for rice and potato (see below).

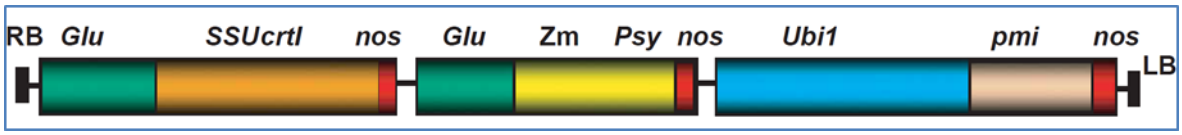
5.5.1.1 Molecular characterisation - Golden Rice 2

Golden Rice 2, a β-carotene enriched rice, is based upon Golden Indica and Japonica rice lines, the latter two having been designed to comply with common regulatory requirements in the EU (Hoa et al. 2003). The original transgenic rice line showing an increased ß-carotene phenotype ("Golden Rice 1") had multiple inserts and relied on antibiotic resistance marker genes for the selection of transgenic transformants and could not be pursued for marketing purposes (Ye et al. 2000). As Golden Indica and Japonica rice lines provided insufficient amounts of ß-carotene (1.6 µg/g dry weight) they were subsequently abandoned for commercialisation. Therefore, only the molecular characteristics of Golden Rice 2 are discussed in detail.

Construction and transformation of the transgenic insert of Golden Rice 2 (Paine et al. 2005)
As major limiting factor for the low production of carotenoids in Golden Rice the daffodil phytoene synthase was identified (Paine et al. 2005).

For Golden Rice 2 the daffodil phytoene synthase was replaced by a maize orthologous which was fused to the rice glutelin promoter and combined with the bacterial carotene desaturase *crtI* gene (source*: Erwinia uredovora*) used already in Golden Rice (see Figure 12). Both were cloned into the T-DNA region of vector pSYN12424. The *crtI* gene was under the control of the rice glutelin promoter and was fused to the chloroplast transit peptide sequence of the small subunit of the ribulose bisphosphate carboxylase gene from peas. The selectable marker cassette contained a phosphomannose isomerase gene under the control of a maize polyubiquitin promoter. Selection of transgenic plants was facilitated on media containing mannose as sole carbon source. It is noteworthy that the construct contained no introns because it was shown that they did not have had any effect on carotenoid accumulation. 619 primary transformants were produced with pSYN12424 by *A. tumefaciens*-mediated transformation of rice cultivar Asanohikari. Quantitative PCR was used to detect events which retained only a single copy of the insert. Due to inherent inaccuracies of the PCR method a small proportion of the identified 103 single copy events may have been incorrectly categorised.

Figure 12: Schematic representation of the T-DNA in vector pSYN12424 used for the production of Golden Rice 2 (Paine et al. 2005)



Glu: rice glutelin promoter
SSU: chloroplast transit peptide of the ribulose-bis-phosphate carboxylase small subunit
crtI: bacterial phytoene desaturase
nos: nopaline synthase terminator
Zm Psy: Zea mays phytoen synthase
Ubi1: maize polyubiquitin promoter
pmi: phosphomannose isomerase
RB, LB: right, left border of T-DNA

There was no evidence to suggest that plant phenotype, seed weight or germination were affected by the presence of the transgenes, however the developers emphasised that further research and development activities should be done before these events could be authorised for commercial use (Paine et al. 2005).

Expression and stability (Paine et al. 2005)
Expression levels of the inserts were indirectly established by spectrophotometric quantification of carotenoids with RP-HPLC. T2 seeds of homozygous plants (selected by a Mendelian segregation ratio of 3:1 (yellow:white), typical for a single locus insert, and a yellow endosperm color) showed a carotenoid content of up to 37 µg/g dry weight. This was a 23-fold increase compared to the original Golden Rice line.

Stability of the carotenogenic trait was checked only for two generations (T1, T2). This is insufficient and has to be extended, according to the relevant regulations (i.e. at least to five generations) (EFSA 2011a; EC 2013a). Sequence data of the genomic regions flanking the transgenic insert are required. Expression levels of the involved transgenic enzymes (PSY and CrtI) have to be determined in several tissues either by quantification of the proteins (western blots, HPLC) or quantitative RT-PCR of the corresponding transcripts to fulfil basic regulatory requirements for the molecular characterisation of the product before marketing approval can be obtained (EC 2013a).

5.5.1.2 Molecular characterisation - potatoes with the "golden" tuber phenotype

For the development of potato tubers with enhanced ß-carotene levels three major strategies have been pursued over the past few years (Diretto et al. 2010):

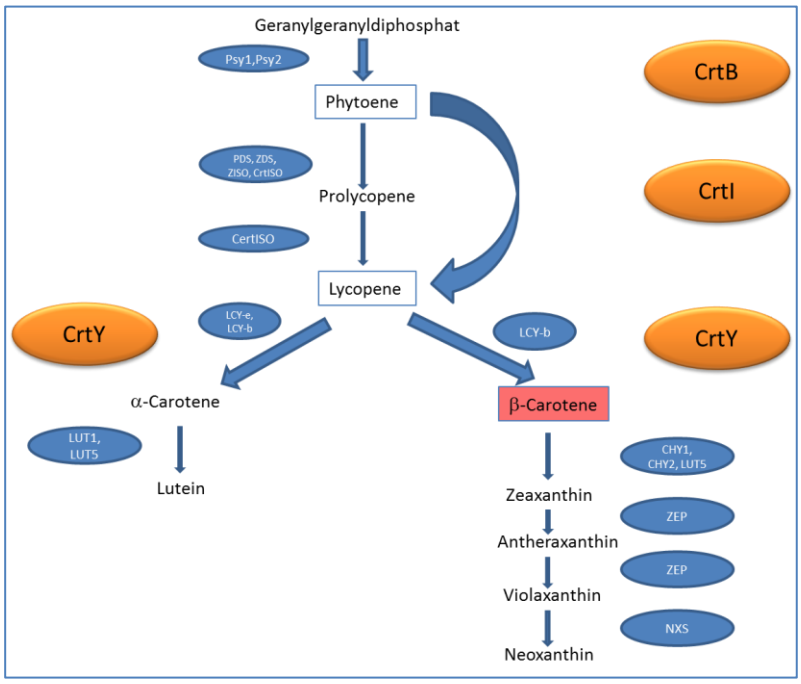
i) "Blocking" strategy: Key biosynthetic steps responsible for the metabolisation of ß-carotene, zeaxanthin and lycopene were blocked by posttranscriptional silencing of the relevant enzymes CHY hydroxylase (conversion of ß carotene to zeaxanthin) (Diretto et al. 2007b; Van Eck et al. 2007), ZEP epoxidase (metabolisation of zeaxanthin) (Romer et al. 2002), and LCY-e cyclase (conversion of lycopene to α-carotene. Down-regulation of LCY-e cyclase leads to an increase of the ß-carotene precursor lycopene) (Diretto et al. 2006). For a schematic representation of the carotenoid biosynthetic pathway in potatoes and the involved enzymes see Figure 13.

ii) "Pushing" strategy: Biosynthetic genes under the control of constitutive or tuber-specific promoters resulting in the increased production of β-carotene, or ß-carotene precursors were overexpressed. This included the overexpression of single genes (e.g. *crtB* (Ducreux et al. 2005), ß-carotene ketolase (Morris et al. 2006)) or of combinations of two or three genes (= mini-pathways) (Gerjets and Sandmann 2006; Diretto et al. 2007a).

iii) "Sink engineering" strategy: This strategy relied on the overexpression of regulatory genes responsible for the differentiation of chromoplasts acting as storage vehicles for carotenoids which were then accumulated in these organelles (Lopez et al. 2008).

The tissue specific overexpression of a mini-pathway (= "pushing" strategy) containing the bacterial genes *crtB* (synthesis of phytoene), *crtI* (conversion of phytoene to lycopene), and *crtY* (formation of α- and ß-carotene from lycopene; see Figure 13) has been the most promising approach so far leading to the highest ß-carotene levels (47 µg/g dry weight) of all four major staple crops (i.e. maize, rice, wheat, potato) yet achieved (Diretto et al. 2010). For a detailed description of the genetic modification of the potato lines expressing the "golden" tuber phenotype see below.

Figure 13: Carotenoid biosynthesis in potato tubers/leaves (modified from Diretto et al. 2010)

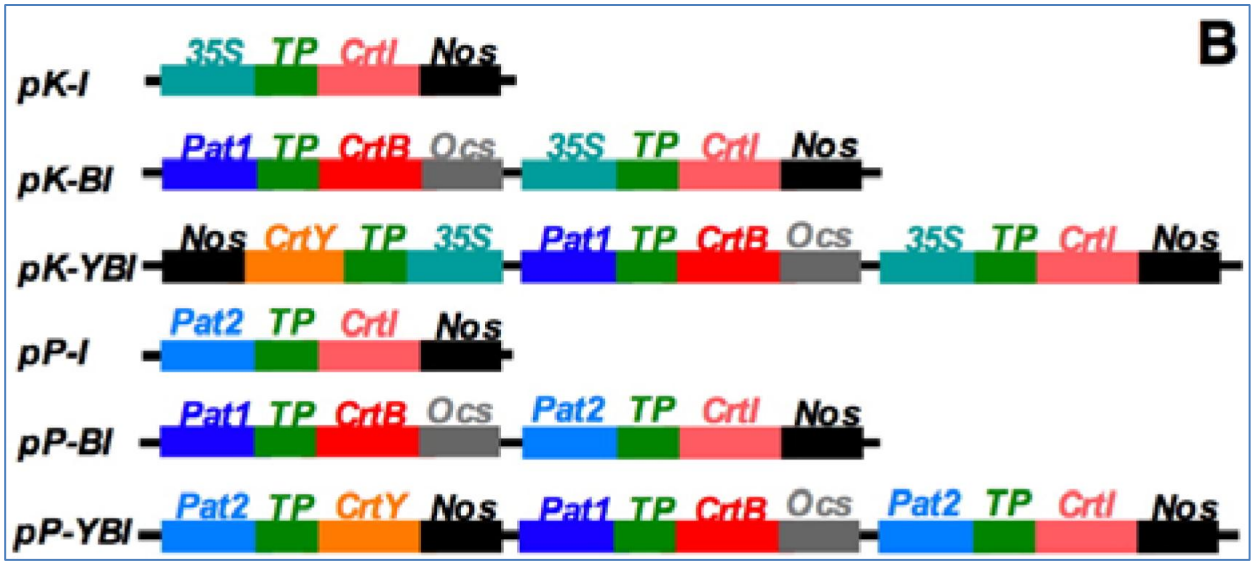


Bacterial enzymes used for plant cell transformation are depicted in orange, plant enzymes in blue.
PSY1,2: endogenous phytoene synthase 1, 2
PDS: endogenous phytoene desaturase
ZDS: zeta-carotene desaturase
ZISO: zeta carotene isomerase
CrtISO: carotene isomerase
LCY-e, LCY-b: lycopene cyclase (epsilon, beta)
CHY1,2: carotene hydroxylase
LUT1, 5: heme carotene hydroxylase
ZEP: zeaxanthin epoxidase
CrtB: bacterial phytoene synthase
CrtI: bacterial phytoene desaturase/isomerase
CrtY: bacterial lycopene cyclase
NXS: neoxanthin synthase (LCY-b paralog)

Construction and transformation of the transgenic insert

To increase the ß-carotene levels in potato tubers three bacterial genes coding for phytoene synthase (crtB), phytoene desaturase (crtI) and lycopene ß-cyclase (crtY) were combined either under the control of a constitutive promoter (35S CaMV) or the tuber-specific patatin promoters (*pat1*, *pat2*) (see Figure 14) (Diretto et al. 2007a). All genes were fused with a DNA sequence coding for the RbcS transit peptide to guarantee a chloroplast localisation of the respective enzymes. The binary vector pCAMBIA1390 served as backbone for the transgenic expression cassettes (Diretto et al. 2007a). *CrtB* and *crtY* were isolated from *Erwinia herbicola*, crtI was a plant codon optimised synthetic version of the bacterial homolog (Diretto et al. 2007a). The potato variety Desiree was transformed with six different promoter-gene combinations (see Figure 3) utilising *A. tumefaciens* (Diretto et al. 2007b). Eighty six transgenic lines could be recovered after selection with kanamycin and were analysed for carotenoid contents in leaves and tubers (Diretto et al. 2007a).

Figure 14: Schematic representation of the transgenic inserts applied to obtain potato tubers with the "golden" phenotype (from Diretto et al. 2007a).



TP: RbcS (ribulose-bis-phosphate carboxylase, small subunit) chloroplast transit peptide
Nos, Ocs: Nopaline synthase and Octopine synthase polyadenylation sequences
35S: Constitutive CaMV 35S promoter
Pat1 and Pat2: Tuber-specific patatin promoters
(pP-YBI appeared to be the most efficient construct and produced tubers with a golden color without any adverse effects on development and other parts of the plant.)

PP-YBI appeared to be the most efficient construct (see Figure 14) producing the highest levels of ß-carotene ever achieved for a major staple crop (Diretto et al. 2007a). The cassettes were highly expressed in tubers but only marginally in leaves. Tubers showed a golden colour, leaves had a normal morphology and colour. There is no information about intactness of the insert, insert localisation in the genome of the potato or the insert copy number available. Due to a lack of relevant information potential risks originating from the molecular characteristics of the event cannot be assessed at the moment. These missing data are a prerequisite before applying for commercial approval (EC 2013a).

Unintended effects were observed with constructs under constitutive 35S promoter control. These constructs generally tended to show lower transformation efficiencies compared to their tissue specifically controlled homologs. Alterations in the macroscopic phenotypes (e.g. abnormal leaf morphology, chlorosis), biochemical composition and endogenous gene expression were obvious with these constructs especially in leaves (Diretto et al. 2007a). CrtB under constitutive control lead to dwarfism of the transformed plants (Diretto et al. 2007a).

Expression of all three bacterial genes were a prerequisite for elevated ß-carotene levels in contrast to the experience obtained with Golden Rice were transformation of already the combination of two transgenes resulted in a significant increase of carotenoids (Paine et al. 2005; Diretto et al. 2007a).

Expression and stability

Expression of the inserted genes was checked by real time RT-PCR and spectrophotometric quantification of pathway metabolites by RP-HPLC. Expression of *crtI* was additionally determined by western blots. The pP-YBI construct was highly expressed in tuber tissue especially in lines 17 and 30 leading to a total carotenoid content of more than 110 µg/g dry weight (Diretto et al. 2010). The phytoene content increased to 19 µg/g but was identified to be still the rate limiting step in the carotenoid pathway (Diretto et al. 2007a). Besides the observed maximum level of 47 µg/g ß-carotene, an increase of α-carotene (which was undetectable in the control) to 6 µg/g was observed. The lutein and violaxanthin levels increased about 30-fold above the wild type levels (Diretto et al. 2007a).

Integration and expression of the pP-YBI construct resulted in the modulation of transcript levels of endogenous enzymes involved in carotene biosynthesis: PSY1, LUT1, NXS were down-, LCY-b, LCY-e, CHY1, and LUT5 up-regulated in transgenic tubers with the Golden phenotype. Additionally, a strong accumulation of phytoene was noticed (Diretto et al. 2010). These observations are indicative for a putatively broad distortion of the plant metabolisms which requires special attention for evaluation potential risks.

Lines 17 and 30 appeared to be outliers compared to the majority of the other lines produced with pP-YBI indicating that the construct itself is not sufficient to establish the golden tuber phenotype (Diretto et al. 2007a).

Transformed potatoes were vegetatively micropropagated *in vitro* and found to be stable for at least two years (= test period). Several different harvests were checked spectrophotometrically and by RP-HPLC showing only minor variations in their carotenoid contents (Diretto et al. 2007a).

Although an impressive amount of expression data has been collected for the tested transgenic potatoes, a detailed characterisation of the expression profiles of the transgenic line ultimately intended for commercialisation is indicative for a comprehensive risk assessment of the product (EC 2013a).

5.5.2 Comparative assessment

In the comparative risk assessment any potential compositional, agronomic and phenotypic difference between the GM plant with a high vitamin content and its comparator needs to be verified. The comparative approach depends on the availability of a conventional comparator (EFSA 2011a; EFSA 2011b).

In relation to the genetic modification of the carotenoid pathway in plants the two potato studies provided the following information regarding significant compositional changes of the GM crop (Diretto et al. 2007a; Diretto et al. 2010):

- Very high total carotenoid levels in tubers were measured for two transgenic potato lines (pP-YBI 17, pP-YBI 30) expressing the three transgenes (*crtB*, *crtI* and *crtY*). The levels were approx. 20-fold compared to non-transgenic control lines (wild-type potato).
- Extremely high ß-carotene levels in tubers were measured for the transgenic "golden" tuber potato line pP-YBI 17. They increased approx. 3600-fold, to 47 µg/g dry weight (DW).
- Tubers expressing the *crtB* (phytoene synthase) and *crtI* (phytoene desaturase) transgenes showed large perturbations in the transcription of endogenous carotenoid genes resulting in the repression of early carotenoid transcripts.
- In the transgenic potato tubers, phytoene increased up to 19 µg/g dry weight. In contrast, phytoene was undetectable in the wild type line.
- Tubers of plant line pP-YBI 17 contained 6 µg/g DW α-carotene. α-Carotene was undetectable in the wild type line. In the tubers also the xanthophyll level (lutein and violaxanthin) went up to 30-fold.
- Combined expression of CrtB and CrtI led to reductions in leaf ß-carotene and ß-xanthophyll contents, and the concomitant increase of leaf α-xanthophyll.
- The expression of CrtB, CrtI and CrtY (ß-cyclase) repressed the transcripts of leaf xanthophyll biosynthesis enzymes.

Taking into consideration the substantial modifications in the carotenoid pattern of the GM potatoes, it can be assumed that a comparative assessment, according to current EFSA standards, could not be conducted, since no adequate comparator could be identified. This depends on whether a similar food and feed can be produced from a non-GM comparator (non-GM potato) with a well-established history of safe use. In this case, a comprehensive safety and nutritional assessment of the GM plant and derived food and feed needs to be carried out (EFSA 2011b).

If a comparative assessment is carried out, which depends on the abovementioned argument, such an comparative approach needs to include all metabolites of the carotenoid pathway measured in all plant tissues (especially in tubers and leaves). Potentially, all products of the isoprenoid pathway should be included in the comparative analysis.

The isoprenoid pathway has been found to be affected by the overexpression of a bacterial phytoene synthase (CrtB) in transgenic oilseed rape with high ß-carotene content. Particularly, the tocopherol levels and the chlorophyll levels were concerned. These GM plants showed, as an intended effect, a 50-fold increase in carotenoids. But, they also showed a decrease of tocopherols and chlorophyll, which are isoprenoids that are also derived from GGPP, like ß-carotene.

In addition, differences in the fatty acid pattern as compared to non-GM control oilseed rape lines were observed. The transgenic rapeseeds contained higher percentages of oleic acid, but lower percentage of linoleic and linolenic acid, which was highly unexpected. As potential reason for these differences, the authors mentioned the stoichiometric increase in double bonds needed to generate the carotenoids, but noted that further experiments are needed to make reliable statements (Shewmaker et al. 1999).

No evaluable data on compositional or phenotypic differences observed in comparison studies for "Golden Rice" are available in scientific literature. Paine et al. (2005) found no evidence to suggest that plant phenotype (seed weight or germination) in the GM rice was influenced by the genetic modification process, but showed no further data. Another study referred to the fact that particular attention should be paid to the pool of compounds associated with carotenoid biosynthesis because this was the pool of metabolites most likely to have been affected by genetic engineering (Chassy 2010).

In conclusion, the design of GM plants with high ß-carotene content and genetically modified carotenoid pathways using biosynthetic enzymes may have unwanted consequences for the synthesis of other metabolic products as e.g. α-carotene, xanthophylls, tocopherol, fatty acids. The comparative approach, therefore, needs to be adapted and the range of compounds extended. The inclusion of metabolites of the targeted pathways in the vitamin enhanced GM plants is highly recommended. In the case of transgenic plants with high ß-carotene content, the metabolites of the isoprenoid pathway have to be included in the comparative assessment.

5.5.3 Toxicological and allergological assessment

Vitamin A is a group of unsaturated nutritional organic compounds that includes retinol, retinal, retinoic acid, and several provitamin A carotenoids, among which β-carotene is the most important. Vitamin A has multiple functions: it is important for growth and development, for the maintenance of the immune system and good vision.

Regarding the possible toxicity, since vitamin A is fat-soluble, disposing of any excesses taken in through diet takes much longer than with water-soluble B vitamins and vitamin C. This allows for toxic levels of vitamin A to accumulate. The acute toxic dose of vitamin A is 25,000 IU/kg, and the chronic toxic dose is 4,000 IU/kg every day for 6-15 months. β-carotene is converted to retinol but not rapidly enough for acute toxicity (Medscape

2013). In people with renal failure, 4,000 IU/kg can cause substantial damage. Children can reach toxic levels at 1,500 IU/kg of body weight (Penniston and Tanumihardjo 2006).

An intake of 3 mg vitamin A (= 10,000 International Units (IU)) per day is assumed non-hazardous for adults (D-A-CH Referenzwerte 2012).

Existing evidences from human trials indicates that supplemental β-carotene (20 mg/day or more) is contraindicated for use in current, heavy smokers. However, there is insufficient scientific basis to set a precise figure for an upper level (UL) of isolated β-carotene as no dose-response relationship for β-carotene effects is available either from the intervention trials in humans or from appropriate animal models. Moreover, it is not possible to be more specific in distinguishing different isomeric forms of β-carotene or specific formulations (EFSA 2006d).

As further described in Chapter 5.5.5, the assessment of the maximum intake scenarios for "Golden Tuber" and "Golden Rice 2" showed no exceedance of the recommended daily intake of retinol-equivalent, -and for the minimum intake scenarios the recommended retinol-equivalents could not even be reached, in all consumer groups.

However, it is well imaginable that vitamin enhancement using genetic engineering will become important in the production of dietary supplements, although such supplementing is not recommended for most of the vitamins for healthy adults. In this case much higher doses/concentrations of ß-carotene would be possible, coming into the range where ß-carotene provokes adverse effects. There are findings that suggest that ß-apocarotenoids function as naturally occurring retinoid antagonists. The antagonism of retinoid signalling by these metabolites may have implications for the activities of dietary ß-carotene as a provitamin A and as a modulator of risk for cardiovascular disease and cancer (Eroglu et al. 2012).

Coming to allergology, no data on sensitising effects of ß-carotene were available. Even in carrot allergic individuals, ß-carotene has not been linked to allergenicity. Actually, ß-carotene seems to act allergy-protective through its *in vivo* anti-oxidative effect (Neuman et al. 1999).

No evaluable data on toxicological and allergological risk assessments are available for "Golden Rice", and also Diretto et al. (2010) did not perform toxicity studies with the high β-carotene potato tubers. However, with respect to potential unintended effects due to the genetic modification and associated toxicological and allergological risks of GM plants with high β-carotene content, the following tests should be conducted: Newly expressed proteins (e.g. microbial phytoene synthase CrtB): biochemical characterisation, digestibility tests, heat stability, bioinformatics analysis, 28-day repeated dose toxicity studies with the newly expressed proteins (in single and combined administration). Whole GM plant: subacute 90-day oral toxicity studies. Additionally, it needs to be verified that any products derived from the GM plants bear no risks for causing long-term adverse effects or adverse effects to development and reproduction systems.

5.5.4 Risks associated with pleiotropic effects

Different mechanisms during plant genetic engineering can cause unintended or pleiotropic effects: novel fusion proteins or ORFs, interruptions of genes, changes at transcriptional or translational level, metabolic shifts, changes in genetic regulatory mechanisms, etc.

Unintended effects have been observed during genetic engineering of GM plants for high vitamin traits. Diretto et al. (2010) revealed that the genetic modification resulted in transcriptional alterations in the carotenoid pathway. Furthermore, transcriptional perturbations as a side-effect of the genetic manipulation of

the carotenoid pathway in potato tubers took place. These alterations were detected by transcriptomic analysis using modern microarray technology (Kloosterman et al., 2008).

These effects were said to be likely due to transcriptional perturbations in the metabolic pathways of glycolysis, starch synthesis, lipid biosynthesis and degradation, and also the biosynthesis of secondary metabolites. It was found, however, that the frequency of transcriptional alterations in those pathways is substantially lower than in the carotenoid pathway (Diretto et al. 2010).

Additional unintended effects were observed in the vitamin enhanced potatoes: Levels of expression in leaves were measured for CrtI and CrtY under the control of a *Pat* promoter. These results suggested that *Pat* promoters were not 100% tuber specific but allowed low levels of expression in leaves as well (Diretto et al. 2010).

High variations in the carotenoid content between individually transformed T2, T3, and T4 lines was observed in GM maize with high vitamin A content pointing to unintended changes in plant metabolic pathways: T3 seeds had surprisingly low levels of provitamin A as compared to the T2 and the T4 lines. It was, furthermore, found that variability is manifested not only in different generations but also in individual seeds of maize from a single ear, and suggested that this unintended effect could be due to the germplasm used for transformation and/or to epigenetic effects (Aluru et al. 2008). Similar observations were reported for transgenic rice (Paine et al. 2005).

Genetic modification of citrus plants causing an overexpression of a bacterial *crtB* gene derived from *Erwinia herbicola* in the plant genome, a design for vitamin enhancement in plants that was developed by Hauptmann et al. (1997), was carried out by a Chinese research team: Variations of carotenoid accumulation were observed affecting mainly the xanthophyll metabolism. This resulted in lower violaxanthin contents than in wild type citrus plants, a phenomenon likely caused by feedback inhibition of the metabolic flux (Cao et al. 2012).

In conclusion, and taking into consideration the study results in relation to unintended effects, it is very important to prove that the inserted sequence results only in the intended changes at RNA or metabolite levels. Modern transcriptomic analysis using microarray technology is an appropriate tool providing useful information for the risk assessment of nutritionally enhanced GM crops as e.g. plants with high vitamin content. Appropriate animal feeding studies for evaluating and investigating unintended effects in GM plants with increased vitamin levels should be realised.

5.5.5 Exposure assessment

5.5.5.1 Introduction

Second generation GM plants have altered ingredients and altered properties and it is necessary to assess the risk of these transgenic organisms and derived new food products. In this chapter, an exposure assessment of GM plants with high amounts of β-carotene is presented.

Plants like leafy green vegetables, orange and yellow vegetables, tomato products, fruits and some vegetable oils are the major sources of provitamin A (National Institutes of Health 2013). Of all carotenoids, β-carotene has the highest provitamin A activity and accumulates naturally for example in carrots, apricots and peaches (Diretto et al. 2010).

Staple food like rice, wheat and potato do not provide provitamin A, so the way of thinking is to modify staple food like rice or potatoes genetically to express a high amount of β-carotene (Paine et al. 2005).

The target groups are populations with a poor supply of vitamin A. Worldwide over 250 million people are affected of vitamin A deficiency (VAD) which is one of the most prevalent nutritional deficiencies in developing countries (Aluru et al. 2008). Especially in Asia, vitamin A deficiency is associated with the poverty related predominant consumption of rice, which lacks pro-vitamin A in edible part of the grain (endosperm) (Paine et al. 2005).

Hence, vitamin enhancement in GM plants are mainly designed and developed for the commercialisation and the market releases other than the EU market, as e.g. Africa and Asia. In developing countries, vitamin A deficiency typically begins during infancy, when infants do not receive adequate supplies of colostrum or breast milk. In these countries chronic diarrhoea also leads to excessive loss of vitamin A in young children, and vitamin A deficiency increases the risk of diarrhoea. In young children and pregnant women the most common symptom of VAD is xerophtalmia, the early signs are night blindness or the inability to see in low light or darkness. People with a low level of vitamin A often tend to a low level of iron as well, which can lead to anaemia. VAD also increases the severity and mortality risk of infections (particularly diarrhoea and measles) even before the onset of xerophtalmia. According to the World Health Organisation (WHO), 190 million preschool-aged children and 19.1 million pregnant women around the world have a serum retinol concentration below 0.70 µmol/l. This is strongly associated with health consequences during periods of high nutritional demand, such as during infancy, childhood, pregnancy and lactation (National Institutes of Health 2013).

According to the "Austrian Nutrition Report 2012", the intake of retinol-equivalent (RE) in girls and boys (7 - 9 years) is adequate. Only 33.4% of women and 18.2% of men had a sufficient plasma concentration of β-carotene. It is further noticeable that women possess a better status of β-carotene than men, which is caused by a higher consumption of fruits and vegetables. The plasma-concentrations of β-carotene in elderly people were improvable, only 10% had an adequate level. To increase the status of β-carotene, it is recommended eating plenty of yellow and orange fruits and vegetables, like carrots, apricots, papayas, peaches, mangos and green leafy vegetables (Elmadfa et al. 2012).

In Germany, vitamin deficiency diseases occur extremely rarely among healthy adults. Thus, healthy people who consume a balanced diet are usually well supplied with essential nutrients like vitamins. An exception may be vitamin D, in case of an endogenous synthesis is insufficient (Bechthold et al. 2012).

The exposure assessment is focussed on the intake of β-carotene derived from cooked GM potatoes "Golden Tuber" and the cooked GM rice "Golden Rice 2". The calculations are based on the food consumption database of the Austrian population of the "Austrian Nutrition Report 2008" (Elmadfa et al. 2009).

5.5.5.2 Exposure assessment "Golden Tuber"

Potatoes have been cultivated for more than 2000 years and originate from the highlands of South America. Potato ranks fourth among the staple food after wheat, rice and maize. On 18 million hectares, 293 million tons potatoes are worldwide produced, of which 36% in developing countries. Tubers are rich in vitamin C but poor in provitamin A.

Diretto et al. (2007a) invented a "Golden Tuber" with a β-carotene concentration of 47 µg/g dry weight (dw). This β-carotene concentration refers to the dry weight of potatoes, so a factor was included to simulate the β-carotene concentration in cooked potatoes. Based on the nut.s software (see Chapter 5.1), cooked potatoes contain 0.804 g water/g fw. The dry weight factor is 1 - 0.804 = 0.196. The β-carotene concentration of 47 µg/g is multiplied with the dry weight factor of the cooked potato (47 µg/g * 0.196 = 9.212 µg/g). The β-carotene

concentration of 9.2 µg/g cooked potato was used for the performed exposure assessment combined with the food consumption database of the Austrian population.

It is considered that the consumption of potatoes, for the maximum intake scenario, consists of 100% of GM "Golden Tuber". For the minimum intake scenario, the calculation is performed with the assumption that the potatoes are containing only traces of 0.9% GM "Golden Tuber" (see explanations in Chapter 5.1 "Method used for the exposure assessment").

The food consumption data were taken from the Austrian food consumption database of the Austrian Nutrition Report 2008 (Elmadfa et al. 2009). The exposure assessment was performed for men and women (19 - 65 years) and children (6 - 15 years). Mean and 95th percentile values were calculated over the collective data from men, women and children. The collective is defined as the mean intake of food based on the data collection.

Table 12 illustrates the maximum intake scenario of β-carotene, retinol equivalent and vitamin A IU due cooked potatoes of the Austrian population (men, women and children). The exposure assessment was performed by combining the potato intake with the GM β-carotene concentration of 9.19 µg/g cooked potato. To conclude from the intake of β-carotene via potato to retinol-equivalent, the β-carotene concentration was divided by 6 because 1 mg retinol-equivalent corresponds to 6 mg all-*trans* β-carotene. To convert retinol-equivalent into vitamin A IU, a factor of 3.33 was applied (see Table 14).

Table 12: Intake of β-carotene, retinol equivalent and vitamin A IU due to the consumption of 100% GM cooked potatoes of men, women and children (maximum intake scenario)

Maximum	Men		Women		Children	
100% GM potatoes	Collective		Collective		Collective	
	Mean	P95	Mean	P95	Mean	P95
potatoes (g/d)	55.2	210	51.0	200	39.3	106.5
β-carotene (µg/d)	507.89	1930.77	468.93	1838.83	361.30	979.25
retinol-equivalent (µg/d)	84.65	321.79	78.16	306.47	60.22	163.21
retinol-equivalent (mg/d)	0.08	0.32	0.08	0.31	0.06	0.16
vitamin A (IU/d)	282.16	1072.65	260.52	1021.57	200.72	544.03

P95 = 95th percentile

As demonstrated in Table 12, no consumer groups of collective 95th percentile men, women and children (4 - 10 years) did exceed the recommended daily intake of retinol-equivalent.

Table 13 demonstrates the scenario of the minimum intake of β-carotene due to cooked potatoes in men, women and children. The β-carotene concentration of 9.19 µg/g cooked potato and 0.9% contamination rate of potatoes with "Golden Tuber" were used for the performed exposure assessment combined with the food consumption database of the Austrian population. The 0.9% simulates unavoidable GM contamination (EC 2003) (see explanations in Chapter 5.1 "Method used for the exposure assessment"). The β-carotene value is divided by 6 to compare the results with the D-A-CH reference values of retinol-equivalent). The retinol-equivalent was multiplied with the factor 3.33 to convert into vitamin A IU (see Table 14).

Table 13: Intake of β-carotene, retinol-equivalent and vitamin A IU due to the consumption of 0.9% GM cooked potatoes of men, women and children (minimum intake scenario)

Minimum	Men		Women		Children	
0.9% GM potatoes	Collective		Collective		Collective	
	Mean	P95	Mean	P95	Mean	P95
potatoes (g/d)	55.2	210	51.0	200	39.3	106.5
β-carotene (µg/d)	4.57	17.38	4.22	16.55	3.25	8.81
retinol-equivalent (µg/d)[1]	0.76	2.90	0.70	2.76	0.54	1.47
retinol-equivalent (mg/d)[1]	0.001	0.003	0.001	0.003	0.001	0.001
vitamin A (IU/d)[2]	2.54	9.65	2.34	9.19	1.81	4.90

[1] 1 mg retinol-equivalent = 1 mg retinol = 6 mg all-trans-β-carotene = 12 mg other pro-vitamin A carotenoids = 1.15 mg all-trans-retinylacetat = 1.83 mg all-trans-retinylpalmitat
[2] 1 IU (In the pharmacology, the "international unit" is a unit of measurement for the amount of a substance) = 0.3 µg retinol
P95 = 95th percentile; m = masculine; f = feminine

There is no evidence of exceeding the recommended daily intake of retinol-equivalent intake via cooked potatoes with 0.9% GM β-carotene contamination in men, women and children of the collective mean and collective 95th Percentile (see explanations in Chapter 5.1 "Method used for the exposure assessment").

Table 14: Recommended daily intake of vitamin A (retinol), β-carotene in mg retinol-equivalent/day (D-A-CH Referenzwerte 2012)

	Retinol-equivalent[1] mg/day	
	Children	
Age	m	f
4 - < 7 years	0.6	
7 - < 10 years	0.8	
10 - < 13 years	0.9	
13 - < 15 years	1.1	1
	Adults	
	m	f
19 - > 65 years	1	0.8

[1] 1 mg retinol-equivalent = 1 mg retinol = 6 mg all-trans-β-carotene = 12 mg other pro-vitamin A carotenoids = 1.15 mg all-trans-retinylacetat = 1.83 mg all-trans-retinylpalmitat
m = masculine; f = feminine

Table 15 shows the approximated intake of cooked Golden Tubers of the Austrian population (men, women and children) to reach the D-A-CH Reference-values. The Austrian population is considered a non-target group for this GM event. The β-carotene concentration of the cooked "Golden Tuber" is about 9.2 µg/g. For example, the D-A-CH Reference-value for men (19 - 65 years) is 1 mg retinol-equivalent (RE) per day, which is about 6000 µg/d β-carotene. Based on this calculation, 552.6 g cooked potatoes must be consumed by men to achieve the D-A-CH Reference-value of β-carotene.

Table 15: Estimated intake of cooked Golden Tubers to obtain the D-A-CH Reference-values

Population group	D-A-CH Reference-value in mg RE/d*	D-A-CH Reference-value in µg/d β-carotene*	Intake of cooked potato in g/d
men	1	6000	652.59
women	0.8	4800	522.07
children (4 - < 7 years)	0.6	3600	391.55
children (7 - < 10 years)	0.8	4800	522.07
children (10 - < 13 years)	0.9	5400	587.33
children (13 - < 15 years) m	1.1	6600	717.85
children (13 - < 15 years) f	1	6000	652.59

RE = retinol-equivalent; m = masculine, f = feminine; * D-A-CH Referenzwerte 2012

5.5.5.3 Exposure assessment "Golden Rice 2"

Rice is for human nutrition the world's most important cereal crop. There is evidence that rice has been consumed for 5,500 years in Thailand, about 5,000 years in India and over 3,000 years in China. Rice consumption varies among countries. For example in Asia 31% of total energy intake originates from rice (country range from 2% to 71%). in Africa 8% of total energy intake (country range 3% to 47%). in Latin America 11% of all food energy (country range 1% - 30%). The consumption of rice is negligible in Europe, Australia, New Zealand and North America (< 2%) (ILSI 2008).

Paine et al. (2005) created the "Golden Rice 2" with a carotenoid amount up to 37 µg/g of which 31 µg/g dw is β-carotene. This concentration is a higher value than in the original "Golden Rice". For the exposure assessment the β-carotene concentration of 31 µg/g dw was calculated with the dry weight factor of cooked rice. Based on the software nut.s, cooked rice contains 0.68433g water/g rice. Following calculation was performed to assess the dry weight factor of cooked rice: 1 - 0.68443 = 0.31557. This factor is multiplied with the β-carotene concentration of 31 µg/g dw and so the β-carotene concentration of cooked rice was estimated (31 µg/g dw * 0.31557 = 9.78267 µg/g).

Table 16 demonstrates the maximum intake scenario of the Austrian population (men, women and children) due 100% GM rice consumption. The exposure assessment referred to the intake of 100% GM β-carotene, retinol-equivalent and vitamin A IU was performed by combining the rice intake with the 100% GM β-carotene concentration of 9.78 µg/g cooked rice. To conclude from the intake of β-carotene via rice to retinol-equivalent, the β-carotene concentration was divided by 6 because 1 mg retinol-equivalent corresponds to 6 mg all-*trans* β-carotene. For converting retinol-equivalent into Vitamin A IU a factor of 3.33 was included (see Table 14).

Table 16: Intake of β-carotene, retinol-equivalent and vitamin A IU due to the consumption of 100% cooked rice GM rice, of men, women and children (maximum intake scenario)

Maximum	Men		Women		Children	
100% GM rice	Collective		Collective		Collective	
	Mean	P95	Mean	P95	Mean	P95
rice (g/d)	28.0	182	23.1	182	14.5	56.4
β-carotene (µg/d)	273.91	1775.55	225.98	1775.55	141.85	551.74
retinol-equivalent (µg/d)	45.65	295.93	37.66	295.93	23.64	91.96
retinol-equivalent (mg/d)	0.05	0.30	0.04	0.30	0.02	0.09
vitamin A (IU/d)	152.17	986.42	125.54	986.42	78.80	306.52

P95 = 95th percentile

The exposure assessment of the maximum intake scenario shows that there is no overconsumption of retinol equivalents based on the collective mean and collective 95th percentile in men, women and children. No other foods containing β-carotene are concluded in this scenario.

Table 17 visualises the exposure assessment of the minimum intake for the Austrian population of 0.9% GM β-carotene via cooked rice of collective mean and collective 95th percentile in men, women and children (see explanations in Chapter 5.1 "Method used for the exposure assessment"). The Austrian population is considered a non-target group for this GM event.

To compare the intake with the D-A-CH reference values, the intake of β-carotene was divided by 6 to convert into retinol-equivalent. To convert retinol-equivalent into Vitamin A IU, a factor of 3.33 was applied.

Table 17: Intake of β-carotene, retinol-equivalent and vitamin A IU due to the consumption of 0.9% cooked GM rice, of men, women and children (minimum intake scenario)

Minimum	Men		Women		Children	
0.9% GM rice	Collective		Collective		Collective	
	Mean	P95	Mean	P95	Mean	P95
rice (g/d)	28.0	182	23.1	182	14.5	56.4
β-carotene (µg/d)	2.47	15.98	2.03	15.98	1.28	4.97
retinol-equivalent (µg/d)	0.41	2.66	0.34	2.66	0.21	0.83
retinol-equivalent (mg/d)	0.0004	0.0027	0.0003	0.0027	0.0002	0.0008
vitamin A (IU/d)	1.37	8.88	1.13	8.88	0.71	2.76

P95 = 95th percentile

Based on the minimum intake scenario, in all consumer groups the recommended reference values of retinol-equivalent were by far not exceeded.

In Table 18 the estimated intake of cooked "Golden Rice 2", to achieve the D-A-CH Reference-values, is demonstrated. The β-carotene concentration of cooked rice is about 9.8 µg/g and for example men have to eat about 613 g cooked "Golden Rice 2" to reach the recommended D-A-CH Reference value of β-carotene.

Table 18: Estimated intake of cooked Golden Rice 2 to obtain the D-A-CH Reference-values

Population group	D-A-CH Reference-value in mg RE/d*	D-A-CH Reference-value in µg/d β-carotene*	Intake of cooked rice in g/d
men	1	6000	613.33
women	0.8	4800	490.66
children (4 - < 7 years)	0.6	3600	368.00
children (7 - < 10 years)	0.8	4800	490.66
children (10 - < 13 years)	0.9	5400	552.00
children (13 - < 15 years) m	1.1	6600	674.66
children (13 - < 15 years) f	1	6000	613.33

RE = retinol-equivalent; m = masculine, f = feminine; * D-A-CH Referenzwerte 2012

To sum up, the intakes of GM β-carotene via "Golden Tuber" and "Golden Rice 2" based on the food database of the "Austrian Nutrition Report 2008" are separately estimated. The intakes of potato and rice of men, women and children of collective mean and collective 95th percentile are calculated using the β-carotene concentration of the cooked GM plants. For the maximum intake scenario, it is considered that 100% of the consumed potatoes and rice are genetically modified. For the minimum intake scenario, it is considered that 0.9% of potatoes and rice are genetically modified (see explanations in Chapter 5.1 "Method used for the exposure assessment").

To evaluate the calculated β-carotene intake, the results are compared with the recommended daily intake of the D-A-CH reference values. Hence, it is necessary to convert β-carotene into retinol-equivalent.

The exposure assessment of a maximum intake scenario based on "Golden Tuber" and "Golden Rice 2" shows no exceedance of the recommended daily intake of retinol-equivalent in the group collective 95th percentile in men, women and children (4 - 10 years). The minimum intake scenario based on "Golden Tuber" and "Golden Rice 2" demonstrates no over estimation of retinol-equivalent in all consumer groups.

Finally, it has to be noted that in the exposure assessment no other β-carotene sources of the everyday diet have been considered. Hence it is supposed that the daily intake of "β-carotene from conventional plants" combined with "β-carotene from genetically modified plants" is higher.

Furthermore, it has to be mentioned that the exposure to heat and light in presence of oxygen causes vitamin A losses. The mean of vitamin loss through preparation of food is established as 20%. The vitamin A activity of the pro-vitamins is reduced even in anaerobe conditions by light or heat, caused by the formation of cis-isomers (D-A-CH Referenzwerte 2012).

However, it is as well possible that vitamin enhancement using genetic engineering could play an important role for the production of dietary supplements, although such supplementing is not recommended for most of the vitamins for healthy adults. An advantage in food processing could be that an industrial fortification of the food would not be needed or could be replaced by genetically modified fortification of plant seeds or grains. The market for vitamin supplemented food in the EU definitely exists, even when it is proven that unfavourable dietary habits cannot be compensated for by taking vitamin supplements or other dietary supplements (Bechthold et al. 2012).

5.6 GM plants with expression of thaumatin

Thaumatins belongs to a group of high molecular proteins that are derived from plants and are capable of inducing sweet taste responses. At least five different forms of thaumatin that are highly related have been

identified: thaumatin I, thaumatin II, thaumatin III, thaumatin b, thaumatin c (Fischer et al. 1998). The sweet proteins thaumatins are about 3000 times sweeter than sucrose. They are used as flavour enhancers and low-calorie sweeteners. Conventionally, they are produced from a tropical monocot, native to West Africa, *Thaumatococcus daniellii* Benth (Stoger 2012; Szwacka et al. 2012).

An advantage of the low-calorie thaumatin proteins lies in the fact that they may be used as natural sweeteners by people trying to reduce excessive consumption of high-calorie foods.

While thaumatins are usually extracted from natural sources and used as food or feed additive, genetic engineering of plants for thaumatin production could find new ways of marketing: the growing, trading, and consumption of fresh transgenic vegetables or fruits. Another, more economically, aspect could be that the direct secretion of the thaumatin proteins from the plant (e.g. hairy roots) into the medium reduces the cost of purification. Additionally, relatively simple reactor designs are needed to produce thaumatin on large scales (Stoger 2012).

The expression of thaumatin is usually induced by insertion into the plant genome of a gene cassette bearing a pre prothaumatin II gene encoding for a precursor of the thaumatin II protein. Several experiments with thaumatin expressing transgenic plants have been carried out concerning different crop plants as follows: potato, cucumber, tomato, pear, strawberry, tobacco (Szwacka et al. 2012). Other fruits and vegetables like e.g. apple, orange, banana, plum, carrot, lettuce, and sugar beet are also in the focus of research for transgenic thaumatin production (Fischer et al. 1998).

The evaluation of the most important aspects on the risk assessment of GM plants producing thaumatin focusses on experiments and studies of Szwacka et al. (2012, 2002) covering thaumatin II gene plant expression systems.

5.6.1 Molecular characterisation

5.6.1.1 Molecular characterisation of cucumber plants containing the thaumatin II gene

In this chapter, transgenic cucumber plants with an expression cassette containing the preprothaumatin II gene as presented in Szwacka et al. (2002) are described.

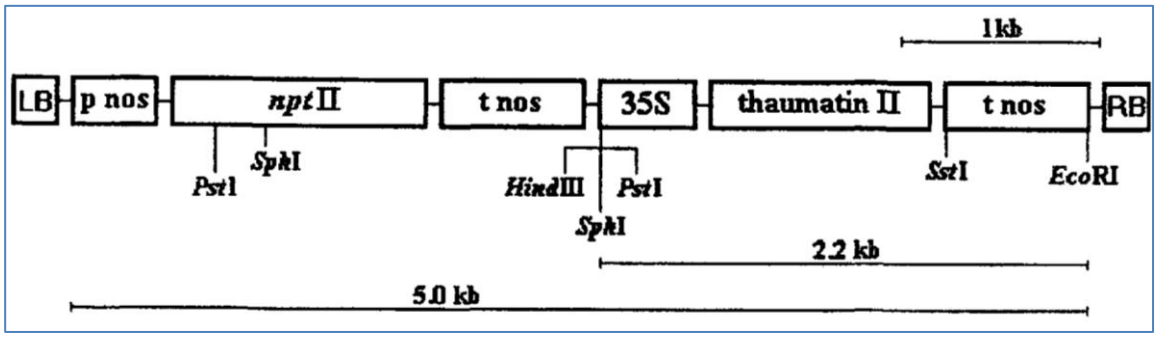
Construction and transformation of the transgenic insert
Leaf fragments from cucumber plants (*Cucumis sativus* L cv. Borszczagowski) were transformed with the binary vector pRUR528 using *Agrobacterium tumefaciens*. The plasmid contained the preprothaumatin II cDNA under the control of the CaMV 35S promoter and terminated by the nopaline synthase terminator (nos) sequence (see Figure 15).

Selection was performed with an aminoglycoside phosphotransferase gene (*nptII*) controlled by the nos promoter and the t nos terminator located on the same plasmid in medium containing kanamycin (Szwacka et al. 2002).

Transformants were obtained with one, two or five integration sites in diploid transgenic cucumber plants. Transformants showing a 3:1 Mendelian ratio and normal morphologies and viable seeds were used for further analysis. This approach resulted in 13 T2 plants representative for 5 parent plants (lines 210, 212, 215, 254, and 225). Presence of the transgenic expression cassettes (nptII and thaumatin II) was confirmed by Southern blots using HindIII and EcoRI/HindIII restriction and hybridisation with thaumatin- and nptII-specific probes. The probes did not overlap nor cover the whole transgenic insert. Tests for absence of vector backbone sequences were not performed. Southern blot results were confirmed by PCR. Gene expression

levels were determined by semi-quantitative Northern blots using densitometry of the obtained banding patterns in T1 and T2 plants. Expression of the thaumatin II gene was determined in T2 plants using SDS PAGE based immunoblots (Szwacka et al. 2002).

Figure 15: T-DNA region of vector pRUR528 (from Szwacka et al. 2002)



The length of the respective restriction fragments are indicated in kilobasepairs.
RB, LB… right and left borders of the T-DNA
p nos… nos promoter
nptII…. kanamycin resistance
t nos… nos terminator
35S… CaMV promoter region
thaumatin II…preprothaumatin II cDNA

Expression and stability of the thaumatin II trait

Inter- and intra-variabilities in the expression patterns of the thaumatin II gene were observed in the obtained transformants. The variability was independent from the copy number of the genomic T-DNA inserts. Variations in thaumatin II protein accumulation levels in the ripe fruits were obvious. However, there was no correlation between protein and mRNA levels observed, suggesting that thaumatin may be controlled both transcriptionally and translationally. It is noteworthy that mRNA levels obtained by Northern blots are imprecise and produce only a vague impression about the actual amounts of the generated mRNAs. These data have to be confirmed by real time RT-PCR.

The thaumatin level in leaves was low compared to a significant expression in the fruit in certain transgenic cucumber lines.

Stability of the thaumatin II trait was confirmed by immunoblots for only three generations (T1, T2, and T3). Genetic stability was tested for two generations (T1, T2). For a proper risk assessment the data for at least five generations must be available (EFSA 2011a).

The transgenic construct contains an antibiotic resistance marker gene (*nptII*), which should be avoided, according to current EU regulations (EC 2013a).

5.6.2 Comparative assessment

In the risk assessment of thaumatin producing GM plants, special emphasis should be placed on the comparative assessment between the transgenic plant and its non-GM comparator aiming at detection and evaluation of intended and unintended effects. A number of results of different comparative studies with thaumatin producing GM plants are available showing the following specifications (Szwacka et al. 2012):

- Different metabolic profiles were observed in GM cucumbers events with different integration sites resulting in significant differences in the levels of 38 compounds as compared to the controls.

- Leaf analysis showed significant alterations of total soluble phenolics and lignin, and also precursors of phenolic compounds were concerned and showed changed concentrations.
- Interestingly, transgenic cucumber lines with relatively high contents of thaumatin showed low contents of total soluble protein in leaves and fruits. A decrease of the amino acid phenylalanine was also observed.
- Aroma differences were detected: higher levels of the main cucumber fruit odorant (E,Z)-2,6-nonadienal were measured in the transgenic lines.

For some of the observed significant differences, it is said that the results were still within the normal range for both the transgenic and control fruits indicating that the observed changes are of no biological relevance. But, it has to be said that the data available from the scientific literature do not allow for a detailed analysis of the comparative tests carried out with the thaumatin producing cucumbers. Especially, the following information is missing or fragmentary: locations of the field trials, raw data, single site results, statistical formulae, environmental conditions, pesticide usage.

The available data on the comparative analysis permit the statement that there are indications the thaumatin expression in GM cucumber has a direct influence on the protein fraction in the cucumber fruits: the expression levels of thaumatin are relatively high in those fruits that showed low soluble protein concentrations. The levels of other components (e.g. the reducing sugars glucose and fructose), however, seem to be altered independently of the thaumatin contents measured in the fruits.

An increased lignification of the fruit cell walls was also measured for the transgenic cucumbers with high thaumatin levels. Increasing of lignin levels can influence digestibility in mammals, including humans.

A thaumatin II gene was also expressed in transgenic tomato events leading to compositional changes in tomato fruits as well. These alterations concerned fruit nutrients and mineral components, and also lycopene, which is a natural carotenoid of high nutraceutical value: it protects the cells from oxidative damage and supports the activity of anti-oxidative enzymes like superoxide dismutase, glutathione peroxidase, or glutathione reductase. The lycopene values in the tomato fruits were found to be outside the normal range for both the transgenic and control tomato fruits, and between the GM line and the control line significant differences were observed (Kosieradzka et al. 2014).

In conclusion, and based on the available scientific information, a comparative assessment is applicable for transgenic plants producing thaumatin proteins and would provide significant data if performed according to current guidance (EFSA 2011a; EC 2013a) and by selection of endpoints mentioned in the respective OECD consensus document. In case of thaumatin producing GM tomatoes: OECD 2008. These endpoints consist of: proximates, minerals, fatty acids and phytosterols, proteins and amino acids, vitamins and other anti-oxidants (e.g. lycopene), and other metabolites (e.g. malic acid, citric acid). Tomato toxicants like steroidal glycoalkaloids, calystegine alkaloids or nicotine should also part of a comprehensive comparative analysis.

5.6.3 Toxicological and allergological assessment

Thaumatin has been studied for its subacute toxicity in rats and dogs and its ability to produce anaphylactic antibodies following oral administration to rats and humans (Higginbotham et al. 1983): "*Thaumatin was readily digested prior to absorption in rats and no adverse effects resulted from its continuous administration to rats and dogs at dietary concentrations of 0, 0.3, 1.0 and 3.0% for 13 weeks. It was not teratogenic when administered orally to rats at 0, 200, 600 and 2000 mg/kg body weight/day from day 6 to 15 of gestation and was without effect on the incidence of dominant lethal mutations when administered on five consecutive days*

to male mice at 200 and 2000 mg/kg/day. The lack of mutagenic potential was confirmed in bacterial mutagenic assays with Salmonella typhimurium (strains TA1535, TA1537, TA1538, TA98 and TA100) and Escherichia coli WP2, at levels of addition of 0.05-50 mg/plate. In rats, thaumatin was found to be a weak sensitizer, comparable with egg albumen, when administered systemically but to be inactive when administered orally. Prick testing of laboratory personnel who had been intermittently exposed by inhalation to thaumatin for periods up to 7 yr showed that 9.3% (13/140) responded positively to commercial thaumatin, while 30.7% were positive to Dermatophagoides pteronyssinus (house dust mite). None of the subjects who gave a positive skin reaction to commercial thaumatin responded to the plant components remaining after removal of the specific sweet Thaumatin proteins. Challenge tests in man did not demonstrate any oral sensitisation. The authors of these studies conclude that the results indicate that thaumatin when used as a flavour modifier and extender, and partial sweetener, is unlikely to be hazardous at the anticipated level of consumption."

JECFA, partly taking into consideration the above-mentioned results, summarises, "*There is no evidence that thaumatin is treated differently than other proteins with respect to hydrolysis or digestion. No antibodies to thaumatin were detected in either rats or humans after prolonged oral administration of quantities of thaumatin that substantially exceed the anticipated human exposure, thus indicating that the intact protein is not absorbed, and confirming the digestibility of thaumatin. The possibility that hormonally-active polypeptides are present in digests of thaumatin, and that these may be absorbed intact and retain their activity, is unlikely because endocrine disturbances were not observed in toxicological studies. Thaumatin showed no mutagenic or teratogenic effects and no allergenic effects were noted. Variations in thyroid weights in a 90-day rat study (increases in males and decreases in females) revealed no treatment-related histological abnormalities; hypo- or hyperthyroid effects were not observed in a follow-up study in which statistically-significant differences in thyroid hormone levels (T3 and T4) were not observed. Slight changes in haemoglobin concentrations, red blood cell counts, and packed-cell volumes observed in rats and dogs fed up to 3.0% thaumatin were not observed in a 13-week clinical study in human volunteers ingesting levels of thaumatin on the order of 140 times higher than the anticipated maximum daily intake, which has been calculated to be 1-2 mg/person/day. The lack of toxicity, combined with its ready digestion to normal food components, indicate that thaumatin's only dietary effect is to make an insignificant contribution to the normal protein intake*" (WHO 1987).

But neither a reference value nor an ADI-level exists for thaumatin. The Scientific Committee on Food SCF agreed that the substance could be considered as acceptable from the toxicological point of view (BfR 2014; Commission of the European Communities 1989). According to its opinion, the acceptability of thaumatin is grounded on its use as an intense sweetener in chewing gums and in flavourings. Existing data gaps were considered to be negligible because of minor consumption of E 957 based on the small spectrum of products where the substance is incorporated. It should be noted, however, that the consumption of thaumatin via a vegetable such as cucumber can be considerably higher.

And so, Szwacka et al. (2012) formulate in an incisive and comprehensible way, "*Although the results of transgenic cucumber and tomato safety evaluations have not shown any negative dietary effects on the health state of model animals, short durations of the feeding studies have given rise to some concern regarding extrapolation of the results to humans. Moreover, since detoxification systems in rodents are considerably different from those in humans in terms of activity and specific detoxification enzymes, there are some additional difficulties in extrapolating the results of animal experiments to humans. To extrapolate the safety of GMO to humans, long-term animal feeding experiments as well as the use of cultured human cell systems are necessary.*"

5.6.4 Risks associated with pleiotropic effects

In conclusion, with respect to thaumatin producing transgenic crops, and also more generally, GM food is often not accepted by consumers because of concerns about unintended effects such as compositional or phenotypical changes not targeted by the genetic engineering process. In this context, Szwacka et al. (2012) pointed out that profiling techniques are considered to be useful strategies for assuring the safety of GM food, but also noted that unintended effects also frequently occur in the course of conventional plant breeding.

The detection of unintended effects leading to adverse effects on humans, animals and the environment, however, is an important issue in GMO risk assessment. Its evaluation includes standard requirements (e.g. field testing, 90-day toxicity tests) and additional studies carried out on a case-by-case basis depending on the genetic modifications introduced into the plant (e.g. the changes in the plant genome). Profiling techniques could provide valuable, additional information on unnatural variations at protein, metabolite or transcript levels. This can help strengthen the risk assessment of second generation GM plants producing novel proteins like thaumatins.

Unexpected and negative consequences for humans due to unintended effects of thaumatin expressing GM plant need to be stringently evaluated using most accurate and reliable test methods. Besides the full utilisation of current requirements (EFSA 2011a; EC 2013a), the focus of an up-to-date risk assessment should be on the realisation of state-of-the-art animal feeding studies, which provide the most reliable data concerning continuous loading of metabolic or effector systems potentially leading to pathologic mechanisms, and the implementation of additional comparison studies based on omics technology.

Omics studies, however, still need to be improved towards a better determination of the consistency of observed differences, and determination of their biological relevance. It is further important that the biological validation of the differences between GM plant and comparator detected by omics technology is verified using adequate statistical methodologies. The inclusion of sufficient biological replicates is an essential element (Ricroch et al. 2011).

5.6.5 Exposure assessment

Intense sweetener must be permitted for their use like other food additives. In the European Union, 11 intense sweeteners are admitted at the moment, like thaumatin (E 957). It is allowed as a sweetener in a few food commodities e.g. in chewing gum and flavourings.

An exposure assessment of the intake of thaumatin from cucumbers (*Cucumis sativus* L.) was performed. Szwacka et al. describe a thaumatin concentration of 4.1 µg/g fw for mature cucumbers. This value was calculated with the Austrian food consumption database which is based on the "Austrian Nutrition Report 2008". The exposure assessment was performed for men and women (19 - 65 years) and children (6 - 15 years). Mean and 95th percentile values were calculated over the collective data from men, women and children. The collective is defined as the mean intake of food based on the data collection. The data were used for the calculation to estimate the intake of thaumatin due to cucumber consumption with such a thaumatin concentration (Elmadfa et al. 2009; Szwacka et al. 2012).

For the maximum intake scenario it is considered that 100% of the consumed cucumbers are genetically modified with thaumatin expression (see Table 19).

Table 19: Intake of thaumatin due to consumption of 100% GM cucumbers, of men, women and children (maximum intake scenario)

Maximum	Men		Women		Children	
100% GM cucumbers	Collective		Collective		Collective	
	Mean	P95	Mean	P95	Mean	P95
cucumbers (g/d)	8.3	50	13.6	84	6.8	33.3
thaumatin (µg/d)	33.93	206.13	55.74	343.56	27.71	136.56

The results of the minimum intake scenario are shown in Table 20, and it is supposed that 0.9% of consumed cucumbers are genetically modified and contain thaumatin (see explanations in Chapter 5.1 "Method used for the exposure assessment").

Table 20: Intake of thaumatin due to the consumption of 0.9% GM cucumbers, of men, women and children (minimum intake scenario)

Minimum	Men		Women		Children	
0.9% GM cucumbers	Collective		Collective		Collective	
	Mean	P95	Mean	P95	Mean	P95
cucumbers (g/d)	8.3	50	13.6	84	6.8	33.3
thaumatin (µg/d)	0.31	1.86	0.50	3.09	0.25	1.23

Based on the provided toxicological information, there is no increased risk due to the intake of cucumber containing thaumatin.

5.7 GM plants producing thermotolerant enzymes

Nowadays, a wide variety of enzymes, which function as catalysts for specific chemical transformations, is used for industrial applications. These include food manufacturing, animal nutrition, cosmetics, and medication. Approximately 200 microbial original type enzymes are currently used commercially including, amongst others, amylases, proteases, lipases, and cellulases (Li et al. 2012a).

As many industrial processing steps often include the use of high temperature (sterilisation of food by heating, baking, dry grinding processes, etc.), research focusses on developing new enzymes with more thermophilic or thermotolerant characteristics. This especially applies to α-amylases, enzymes hydrolysing starch to sugars, which are most prevalent in the industrial enzyme sector. The α-amylases have a wide application spectrum ranging from the production of fuel, distillation processing, the conversion of starch to sugar syrups, and the production of cyclodextrins for the pharmaceutical industry (Syngenta Seeds 2007; Li et al. 2012a).

The importance of thermotolerant α-amylase for industrial applications is reflected in the fact that genetic modification approaches have been used to design transgenic plants producing them. The advantage of such transgenic plants is that the enzymes are produced in the plant tissue (e.g. maize seed) and need not be added separately during the processing step.

The best known such example is GM maize event 3272 expressing the thermotolerant AMY797E protein, a chimeric α-amylase encoded by gene segments derived from three parental α-amylase genes. These genes originate from strains of the archaeal order *Thermococcales* (EFSA 2013a) and are expressed on a high level due to a codon-adapted sequence.

GM maize event 3272 has been authorised in eight countries since 2007 including cultivation purposes for Canada and the United States. It was notified for import and processing and food and feed purposes in the EU

in 2007, but has not yet received a positive decision for commercialisation (CERA 2014). Maize event 3272 was risk assessed by the following different national authorities: USDA, CFIA, FSANZ, J-BCH, and BCH-Pilipinas.

5.7.1 Molecular characterisation

5.7.1.1 Construction and transformation of the transgenic insert

Immature maize embryos were transformed with *Agrobacterium tumefaciens* strain LBA4404 containing the plasmid vector pNOV7013 to generate maize event 3272. Transformed tissue was regenerated after the callus phase. Selection was performed on media containing mannose as the sole carbon source (Syngenta Seeds 2007).

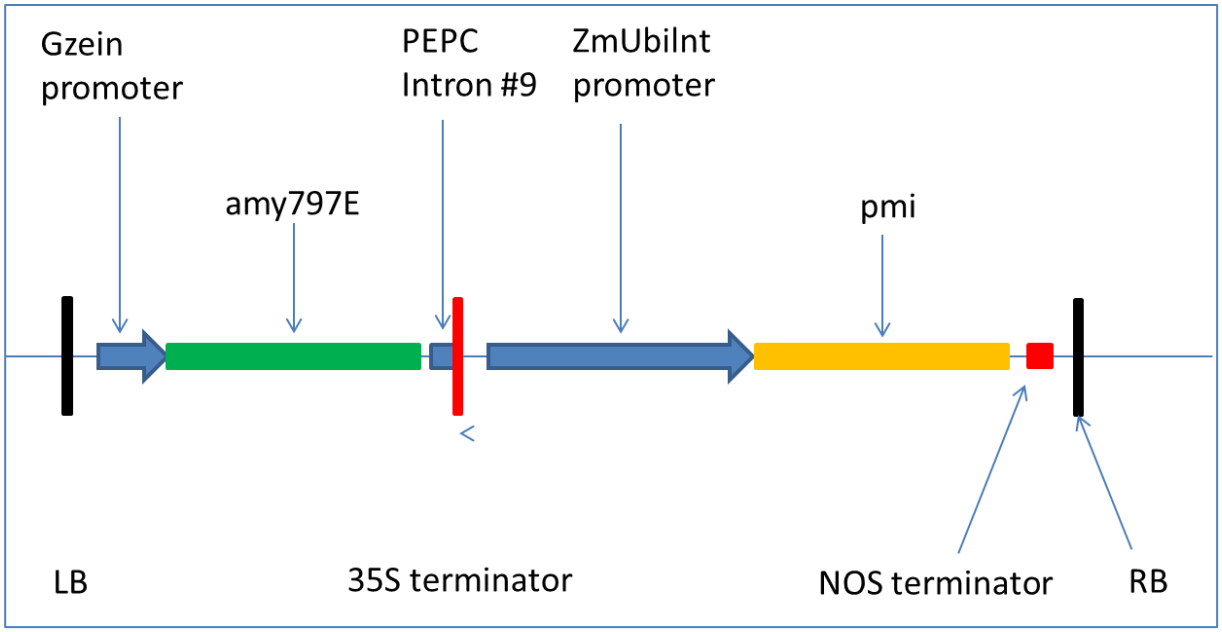
The transformation vector pNOV7013 was carrier for a T-DNA with two expression cassettes inserted between the left and the right T-DNA border sequences (see Figure 16).

amy797E gene expression cassette
The chimeric α-amylase (*amy797E*) gene is under the control of the promoter of a maize γ-zein (=GZein) storage protein and the 35S RNA terminator sequence from the *cauliflower mosaic virus*. A 108 bp fragment of intron 9 of the maize phosphoenolpyruvate carboxylase gene (PEPC9) precedes the terminator sequence. The *amy797E* gene additionally includes a 19 amino acid N-terminal maize γ-zein signal sequence and a C-terminal SEKDEL endoplasmic reticulum retention signal (Syngenta Seeds 2007).

The α-amylase gene *amy797E* was constructed from α-amylase gene templates originating from three thermotolerant archaebacterial strains belonging to the order *Thermococcales*. Two template genes were obtained from pure cultures of *Thermococcus* strains isolated from hydrothermal systems at temperatures of 95°C (pH 7) and 85°C (pH 6) (EFSA 2013a). The third template was obtained from an undetermined number of thermophilic organisms (presumably belonging to *Pyrococcus* or *Thermococcus* species) isolated from deep sea locations in the Pacific Ocean at prevailing temperatures of 90°C (EFSA 2013a). These three bacterial α-amylase templates were selected because of their superior activity under high temperature, low -Ca^{2++} and low pH conditions, which are all advantageous for the starch liquefaction step during maize processing for the generation of bioethanol. The α-amylase coding region of the *amy797E* gene was synthesised to accommodate the preferred codon usage for maize. The final AMY797E protein is 460 amino acids long and carries the maize γ-zein signal sequence as an N-terminal fusion and an endoplasmic reticulum (ER) retention signal as a C-terminal fusion (Syngenta Seeds 2007).

pmi gene expression cassette
The *Escherichia coli pmi* gene encodes a phosphomannose isomerase (PMI) enzyme. The gene is under the control of the promoter and first intron region of the Zea mays polyubiquitin gene and the *NOS* terminator of *A. tumefaciens*. Expression of PMI enables transformed maize cells to utilise mannose, which facilitates survival on media where mannose is the sole source of carbon (Syngenta Seeds 2007).

Figure 16: Gene expression cassettes of the transgenic insert of maize 3272. (Schematic representation modified from Syngenta Seeds S.A.S. 2007)



Gzein: Zea mays 27-kDa storage protein (zein) gene (GenBank® Accession Number X56117; NCBI, 2005). Provides endosperm-specific expression in Zea mays

amy797E: Chimeric, thermotolerant 797GL3 α-amylase gene.

PEPC: Intron #9 from the phosphoenolpyruvate carboxylase gene (GenBank Accession Number X15239) from Zea mays.

ZmUbiInt: Promoter region from Zea mays polyubiquitin gene, contains the first intron (GenBank Accession Number S94464).

pmi: *E.coli* manA gene encoding phosphomannose isomerase (GenBank Accession Number M15380).

35S: Terminator sequence from the 35S RNA from the *cauliflower mosaic virus* genome (Similar to GenBank Accession Number AF140604).

NOS: Terminator sequence from the nopaline synthase gene of *Agrobacterium tumefaciens* (GenBank Accession Number V00087).

LB, RB: Left and right border regions of T-DNA from *Agrobacterium tumefaciens* nopaline ti-plasmid (GenBank Accession Number J01826).

Southern blot analysis demonstrated that maize 3272 contained a single transgenic insert and no vector backbone sequences. Sequence analysis confirmed that the T-DNA insert in maize 3272 was preserved except for the deletion of 23 bp of the 5' right border and 7 bp of the 3' left border. The insert was located in a highly repetitive region - presumably of transposon origin - of the maize genome. No maize endogenous genes were disrupted (EFSA 2013a).

5.7.1.2 Expression and stability of the AMY797E trait

Genetic stability was confirmed by Southern analyses which compared the hybridisation pattern over three generations of event 3272 plants (BC1 to BC3) using the *amy797E* gene as a probe (EFSA 2013a). The hybridisation and PCR data demonstrated that the T-DNA insert from pNOV7013 incorporated into Event 3272 was stable. A Mendelian inheritance pattern of the trait consistent with a single genetic locus was obtained (Syngenta Seeds 2007; EFSA 2013a).

Phenotypic stability of α-amylase and pmi protein expression was studied over multiple generations (backcrosses with maize inbred line NP2222) in grains and leaves under field conditions (EFSA 2013a). Two hybrid lines were tested for expression levels: The mean AMY797E level for grain was 1 259 µg/g dry weight (dw) (range 908-1 562 µg/g dw) for hybrid A and 1 335 µg/g dw (range 893-1 730 µg/g dw) for hybrid B. The mean PMI level for grain was < 0.5 µg/g dw (expressed as the average of quantifiable values; range < LOQ11 to 0.7 µg/g dw) for hybrid A and 0.7 µg/g dw (range 0.5-0.9 µg/g dw) for hybrid B. According to quantitatively

obtained ELISA data, AMY797E α-amylase and PMI appeared to be stably expressed in maize 3272 across multiple generations (EFSA 2013a).

5.7.2 Comparative assessment

In the comparative assessment of the GM maize event 3272, the substantial equivalence between the GM plant and non-GM comparators was statistically tested. In the risk assessment submitted as part of the EU notification, the comparisons were based on non-GM lines which were negative segregants isolated, after backcrossing and selfing of the progeny of the initial transformant. The EFSA GMO Panel, therefore, requested additional compositional and agronomic data including an adequate conventional counterpart, owing to the importance of the comparative assessment for identifying potential unintended effects. Additional data were submitted, but it was found that also these data (derived from field trials performed in 2008) did not fulfil the requirement (e.g. multiple seasons) specified in applicable EFSA guidance, and no conclusion on the comparative assessment can be made (EFSA 2013a).

In contrast, the USDA considered the data presented by the notifier as adequate making the remark that no difference in compositional and nutritional quality of Event 3272 corn compared to conventional corn exists, apart from the presence of AMY797E and PMI. It is, furthermore, argued that none of the values for the forage and grain composition characteristics were outside the range of natural variability of conventional corn as found in the International Life Sciences Institute Crop Composition Database or in the OECD consensus document on corn (USDA 2012).

The Canadian Food Inspection Agency also studied the comparative data generated in the USA in 2004 and pointed out that statistically significant differences were observed between event 3272 and the control lines for ash, protein, carbohydrate, starch, TDF ADF, NDF and TDF, Vitamin B1, B6, most of the amino acids, and ferulic acid (all in grain). It was noted, however, that all levels were within literature values. Based on these findings and the comparative data provided by the applicant, it was concluded that it was unlikely the modification had had any unintended effects on the modified plant, and that the nutritional composition of corn event 3272 was substantially equivalent to conventional corn varieties (Canadian Food Inspection Agency 2008).

The FSANZ commented on the use of negative segregants to serve as non-GM comparators in field testing making the remark that when complex breeding is involved in the development of GM plants intended to be commercialised negative segregants are often the only lines available that are close enough to the GM plant lines to serve as an appropriate control. It is also said that some minor differences in compounds were noted, however the levels observed represented very minor differences and were within the reference range from conventional varieties (Food Standards Australia New Zealand 2007). Similar arguments can be found in the GM maize 3272 risk assessment report of the Philippines Biosafety Clearing-House (2008).

In conclusion, it is important to note that the applicant's comparative data did not comply with EFSA standards that represent the state-of-the-art in European risk assessment in relation to substantial equivalence testing of GM plants at the time of the submission of the application.

The results of the submitted data contains no evidences to suggest that the genetic modification resulted in unintended effects: the composition characteristics were inside the range of natural variability of conventional corn (USDA 2012). However, as the current EFSA guidelines for statistical evaluation of field test data provide a solid basis for comparative testing, additional studies with thermotolerant maize 3272 meeting the EFSA requirements (EFSA 2010c) are needed to draw final conclusions on the safety of the GM plant and derived products.

It is further noted that any GM plants producing thermotolerant enzymes - as long as no substantial modifications are induced (i.e. the GM plant produces an industrial enzyme without being affected at physiological or metabolic level), and as long as appropriate comparators are available (EFSA 2011b) - have to be tested conducting a state-of-the-art comparative risk assessment.

5.7.3 Toxicological and allergological assessment

An acute toxicity test regarding the proteins AMY797E and PMI was performed by the applicant for maize event 3272, with mice getting a single dose of 1500 mg AMY797E7/kg bodyweight (bw) and 3100 mg PMI/kg bw. Under the given conditions no adverse effects could be observed (EFSA 2013a).

On request of the EFSA GMO Panel (EFSA 2013a), also a repeated-dose toxicity test was provided by the applicant, consisting of 3 verum and 2 control groups. Five female and five male Wistar Han rats each received 10,.55 or 550 mg AMY797E/kg bw per day for 28 consecutive days. One control group received only the vehicle (carboxymethylcellulose) and the other one (protein control) received bovine serum albumin (BSA) at a dose of 550 mg/kg bw.

In the intermediate-dose group (55 mg/kg bw), the mean cell haemoglobin concentration (MCHS) in females was higher as against the control. However, this elevation was not observed in the high-dose group. The male rats of the high-dose group had a significantly higher mean thymus weight. These were the only significant effects observed in the experiment, and the Panel did not regard them as toxicologically relevant. The EFSA GMO Panel concluded that no adverse effects are caused by an intake of 550 mg AMY797E /kg bw per day for 28 days in rats (EFSA 2013a).

However, with the given study batteries and designs, no final evidence is possible with reference to long-term (especially appropriate for foodstuffs), reproductive or developmental effects of the whole food and/or feed. Increased attention has to be paid to even very slight deviations from control groups in different parameters. In the present case, not even a 90-day toxicity study was performed.

Coming to potential allergic reactions, in the genetically modified maize 3272 two new sources of recombinant proteins (AMY797E and PMI) are expressed. There is little information about the human exposure to the thermotolerant α-amylase AMY797E from the original microbiological source. Because of the absence of data of *de novo* sensitisation capacity, the EFSA GMO Panel could not conclude on the potential for *de novo* allergic sensitisation of the newly expressed AMY797E protein (EFSA 2013a).

However, there is evidence for the allergenic potential of α-amylase in general. Especially bakery workers frequently show α-amylase-linked allergic reactions, typically in the respiratory tract. Amylase hydrolyses starch, affects the consistency of a dough, and is able to retard staling and is, therefore, utilised in the baking industry. The following organisms are used to produce amylase for food and feed industry: *Aspergillus niger*, *Aspergillus oryzae*, *Bacillus amyloliquefaciens*, *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus stearothermophilus*, *Microbacterium imperiale*, *Trichoderma reesei*, *Trichoderma longibrachiatum* (α-amylase); barley, soybean (ß-amylase) (Martel 2009).

The second protein expressed in maize 3272, the phosphomannose isomerase, originates from *E. coli*. The applicant provided the EFSA GMO panel with additional information about the cross-reactivity of PMI protein to Hev b 13 latex allergen. A bioinformatic analysis was performed and 28% identity with the Hev b 13 protein was shown. A match with the frog allergen α-parvalbumin was described. Allergenicity of PMI was previously assessed in maize MIR604 and MIR162. Based on all the available information, the EFSA GMO Panel considered that there are no indications that the newly expressed PMI protein in maize 3272 may be allergenic.

From the authors' point of view, the applicant should perform animal studies to verify the *de novo* sensitisation potential of the protein (please compare EFSA 2013a). Additionally, further studies including potential long-term and reproductive/developmental effects should be considered.

5.7.4 Risks associated with pleiotropic effects

The risk assessment with respect to unintended or pleiotropic effects of GM maize 3272 resulted in the following observations and conclusions by the risk assessment agencies:

An evaluation by European risk assessment agency EFSA led to the statement that the information from the field trials for the comparative assessment necessary to identify potential unintended effects did not fulfil the current requirements. The EFSA GMO Panel considered the data insufficient in order to exclude the possible presence of unintended effects and no final conclusion on the comparative analysis were drawn. The missing data for identification of unintended effects also resulted in the statement that the EFSA GMO Panel could not conclude on the allergenicity of the whole GM plant (EFSA 2013a).

Regarding potential unintended effects on plant fitness due to the genetic modification, despite the abovementioned data gap, EFSA noted there is very little likelihood that GM maize event 3272 has increased fitness characteristics that will change its persistence and survival following accidental release into the environment of viable grains (EFSA 2013a).

The potential for unintended modifications due to the process of genetic engineering was commented by USDA summing up that data concerning an insertion analysis of the gene construct, gene sequence information about the inserted DNA, genetic inheritance, protein expression, disease and pest resistance characteristics, growth habit, vegetative vigor, reproductive characteristics, yield and grain characteristics, stress adaptation, and nutritional composition of maize event 3272 were submitted to address such unintended effects. The performance of mouse and bird feeding was also mentioned. No adverse effects were identified, and the likelihood of increased production of new allergens, new toxins in the GM maize was considered to be extremely low (USDA 2012).

Other risk assessment agencies made similar comments: Based on the review of the information submitted by the applicant, and through comparisons between GM maize 3272 and non-GM counterparts the Canadian Food Inspection Agency (2008) concluded that the novel genes and their corresponding traits do not confer any characteristic that would result in unintended environmental effects. The Food Standards Australia New Zealand (2007) noted that no public health and safety concerns had been identified in the risk assessment of food produced from event 3272. No safety issues were also raised by the Philippines Biosafety Clearing-House (2008).

In conclusion, it can be said that the available data - taking into consideration the standards and requirements laid down in EU GMO risk assessment guidelines (EFSA 2006a; EFSA 2011a) - do not provide the solid database needed for adequately evaluating potential unintended effects of the GM maize event 3272. Especially, the low number of sites and years of the field trials give reasons for concern. Reliable data for the toxicological and allergological risk assessment caused by unintended effects are also missing (please see Chapter 5.7.3).

5.7.5 Exposure assessment

The intended use of the transgenic maize 3272 is the cultivation outside of the EU and the import for use in the fuel ethanol process. Maize 3272 carries the synthetic *amy797E* gene, which encodes a thermotolerant α-amylase enzyme. For this industrial use, GM and conventional maize are mixed, and because of the presence

of the thermotolerant enzyme in the GM plant there is no need of adding further microbially produced enzymes.

Although this GM maize is to be used in bio-ethanol industry, it cannot be excluded that the crop enters accidentally feed or food (e.g. starch, syrups, maize oil, flakes, coarse and regular grits, coarse and dusted meal, flour, maize germ meal, maize feed, condensed steep water and maize meal) (EFSA 2013a), even though presumably only in small amounts (because of the non-regular but inadvertent entry). But nevertheless, exposure and risk assessments are carried out in the following for all possible food entry scenarios (maximum, minimum consumption; maximum, minimum content).

In the guidance document of the Scientific Panel of Food Contact Materials, Enzymes, Flavourings and Processing Aids (CEF), general principles of risk assessment of food enzymes are described. The following critical issues for the safety assessment have to be considered (EFSA 2009a):

- A consideration of safety issues related to the source of the food enzyme (animals, plants, basidiomycetes or micro-organisms). The possibility of infectious agents in the source, measures for their control in the food enzyme and the potential virulence/ toxicity of the producer organism/micro-organism have to be considered.

- The food enzyme, related to the enzyme protein(s) as well as other constituents, e.g. by-products originating from the source organism and residues of any substances and materials used in the production process.

- Intended and unintended reaction products resulting either from enzymatic or chemical reactions of the food enzyme with food constituents or from the degradation of the food enzyme during storage and processing of the foodstuff.

- The dietary exposure of the consumer. This depends on the residual concentration of the food enzyme(s) and other constituents of the food enzyme in the foods at the time of consumption and the amount and frequency of their consumption.

Each individual food enzyme must be assessed. However, specified food enzymes:

i) with the same catalytic activity (e.g. α-amylase),
ii) produced by the same micro-organism strain and
iii) by the substantially same manufacturing process

may be grouped in one application.

At present, EFSA is evaluating all enzymes from a GM microorganism source which are already used in the food and feed industry.

Although there is an EFSA guidance document for the assessment of food enzymes, the EFSA scientific opinion on GM maize 3272 (EFSA 2013a) did not include an exposure assessment, even though being an essential part of risk assessment (maybe because of the submitted industrial use). Hence, there is only limited information about the release of maize 3272 and the potential human consumption. In the following calculated intakes of corn-products and the possible consequences are described.

The exposure assessment is focussed on the intake of the thermotolerant α-amylase and the marker protein phosphomannose-isomerase. From a field trial with two maize hybrids of 3272 in the USA, the levels of the two proteins (AMY797E and PMI) were determined via enzyme linked immunosorbent assay (ELISA). Hybrid B had the highest maximum concentrations of both proteins (EFSA 2013a). Therefore, minimum and maximum concentrations from hybrid B (Table 21) are used for the exposure assessment.

Table 21: Ranges of AMY797E and PMI in GM maize 3272 in hybrid B

	Levels of AMY797E and PMI	
	GM maize hybrid B min value in µg/g dw*	**GM maize hybrid B max value in µg/g dw***
AMY797E	893	1730
PMI	0.5	0.9

* (EFSA 2013a); AMY797E = thermotolerant α-amylase; PMI = phosphomannose-isomerase; dw = dry weight

Food consumption data were taken from the Austrian food consumption database of the "Austrian Nutrition Report 2008". The following food categories are considered to consist of GM maize 3272 or to contain traces: fresh, steamed and canned maize, maize flakes, -flour, -starch, -bread, -oil and cornflakes. Operating figures of the bodyweights are given in Table 22 (Elmadfa et al. 2009).

Table 22: Operating figures of the bodyweight of the Austrian population

Men	81.5 kg
Women	63.3 kg
Children	39.7 kg

In a maximum intake scenario, these food categories were hypothetically considered to completely consist of maize 3272 containing the thermotolerant α-amylase and phosphomannose isomerase concentrations given in Table 21. The exposure assessment was performed for the collective groups of men and women (19 - 65 years) and children (6 - 15 years) as described in Chapter 5.1 "Method used for the exposure assessment". Results are shown in Table 23. The maximum protein concentration refers to the highest value obtained in GM maize 3272 and the minimum protein concentration belongs to the lowest value.

For the exposure assessment of the minimum intake range of GM maize products with thermotolerant α-amylase, the same food categories were considered to be contaminated with maize 3272 at a level of 0.9%.

Table 24 demonstrates the intake for men, women and children of AMY797E and PMI from maize foodstuffs, all containing 0.9% of 3272. Again, minimum and maximum values of the proteins in maize are given.

Table 23: Intake of AMY797E and PMI due to the consumption of 100% GM maize of men, women and children in mg/kg bw/day (maximum intake scenario)

Maximum	Men		Women		Children	
100% GM maize	Collective		Collective		Collective	
	Mean	P95	Mean	P95	Mean	P95
maize products intake (g/d)	5.38	33	5.74	33	9.5	31.7
sum of AMY797E maximum (mg/kg bw/d)	0.114	0.701	0.156	0.898	0.414	1.380
sum of PMI maximum (mg/kg bw/d)	0.000	0.000	0.000	0.000	0.000	0.001
sum of AMY797E minimum (mg/kg bw/d)	0.059	0.362	0.081	0.464	0.213	0.712
sum of PMI minimum (mg/kg bw/d)	0.000	0.000	0.000	0.000	0.000	0.000

bw = bodyweight; maximum = maximum protein concentration from Table 21, minimum = minimum protein concentrations from Table 21.

Table 24: Intake of AMY797E and PMI due to the consumption of 0.9% GM maize of men, women and children in mg/kg bw/day (minimum intake scenario)

Minimum	Men		Women		Children	
0.9% GM maize	Collective		Collective		Collective	
	Mean	P95	Mean	P95	Mean	P95
maize products intake (g/d)	5.38	33	5.74	33	9.5	31.7
sum of AMY797E maximum (mg/kg bw/d)	0.001	0.006	0.001	0.008	0.004	0.012
sum of PMI maximum (mg/kg bw/d)	0.000	0.000	0.000	0.000	0.000	0.000
sum of AMY797E minimum (mg/kg bw/d)	0.001	0.003	0.001	0.004	0.002	0.006
sum of PMI minimum (mg/kg bw/d)	0.000	0.000	0.000	0.000	0.000	0.000

bw = bodyweight; maximum= maximum protein concentration from Table 21, minimum = minimum protein concentrations from Table 21.

The exposure estimated for the Austrian consumers (Table 23, Table 24) was in all scenarios lower than the lowest dose in the above described sub-acute animal study, i.e. below 10 mg/kg bw per day (EFSA 2013a), as with a rather conservative approach and not that of the EFSA GMO Panel. In the repeated dose toxicity study, in Wistar rats, the described dose of 10 mg/kg bw and day would have to be regarded as no observed effect level. Based on this animal study, a common safety factor of 100 has to be added taken into consideration inter-species and intra-species differences. This results in an acceptable level of 0.1 mg/kg bw. In most of the maximum intake scenarios an exceedance of this value could be observed. With the 0.9% scenario, no value exceeded the acceptable level, at least not this one derived from the submitted tests.

In conclusion, even though maize 3272 is applied and intended for industrial uses, entries into food and feed cannot be excluded, in particular within the permitted range up to 0.9% (see explanations in Chapter 5.1 "Method used for the exposure assessment").

Following a rather conservative approach in judging the two toxicity studies provided by the applicant, the no Oobserved Eeffect level has to be assessed at the lowest dose of 10 mg/kg bw. Taking into consideration the common safety factor of 100 (leading to an acceptable level of 0.1 mg/kg bw and day), no exceedances could be observed in the 0.9% scenario.

Almost all values in the maximum intake scenario exceeded that hypothetical value. Hypothetical, because this value does not include life-time consumption reflecting long-term tests and does not consider reproductive or developmental effects, or other effects that might be seen after prolonged exposure. These factors imply the

necessity to perform further respective studies. Likewise, there are open questions concerning the possible allergenicity of the newly expressed proteins which should be clarified by further studies.

5.8 GM plants with high amounts of lysine

Enhancement of amino acids in GM plants is an important trait developed to provide farm animals with sufficient nutrients. Lysine is essential for humans and animals, and in some feeding crops its amount is too low to provide optimal feed properties, especially for broiler chickens, turkeys and pigs (EFSA 2006c). Especially, the development of lysine enhanced GM plants provides an alternative to the addition of lysine supplements to feeding stuffs. At present, genetic engineering has been used for targeting the production of lysine in a number of crop species including maize, oilseed rape, soybean and rice (Galili and Amir 2013).

The best known example for an increased lysine content is GM maize event LY038, which has been approved in several countries including authorisation for cultivation in the United States, Canada and Japan (ISAAA 2014a). The levels of lysine in GM maize LY038 are enhanced by approximately 40%, which means that the total lysine content in the GM maize is up to 5.3 g/kg dry weight (dw) compared to a maximum value of 2.8 g/kg dw in conventional maize (USDA 2005; Health Canada 2006).

The main genetic principle of the high lysine trait is the introduction of a lysine feedback-insensitive dihydrodipicolinate synthase (cDHDPS) derived from bacteria. In GM maize LY038, its transcription is under control of a maize promoter which leads to the accumulation of lysine in grain. Other ways to construct high lysine trait GM plants are the additional suppression of enzymes (e.g. ketoglutaric acid reductase) that play an important role in lysine catabolism (USDA 2005; Long et al. 2013).

5.8.1 Molecular characterisation

The risk assessment documents submitted by the applicant in a United States petition and evaluation by USDA (2005) provides the most useful information on the molecular characterisation carried out with GM maize event LY038:

Southern blot analyses of the transgenic elements inserted and of the genetic elements from the vector (Plasmid PV-ZMPQ76) not inserted into the maize genome were performed. These analyses were carried out digesting of genomic DNA from LY038 and a negative segregant control with *Nde*I or the combination of *Nde*I and *Nco*I. The gene fragments were separated by gel electrophoresis before the transfer to a nylon membrane for Southern analysis. Additional tests by digestion of the test DNA with restriction enzymes *Spe*I, *Xho*I and *Xba*I were conducted for proving the intactness of the inserted *cordapA* gene cassette.

It was verified that no unexpected genetic elements are present in LY038, and that the GM maize does not contain additional promoter, intron or coding elements other than those associated with the intact cordapA cassette.

A Cre/*lox* recombination system was used to remove the *nptII* antibiotic resistance marker present in plants generated from the initial transformation. A number of different Southern blots were examined with the LY038 DNA, the CaMV e35S promoter, the *nptII* coding region, and the NOS 3' polyadenylation sequence. The results supported the conclusion that the *nptII* cassette and associated partial or intact genetic elements are absent in the GM maize event LY038.

The absence of T-DNA and Plasmid PV-ZMPQ76 backbone sequences were proven by additional Southern blot analyses using probes spanning the entire *cre* gene cassette. A linear plasmid containing the *cre* cassette was

used as positive control DNA. Moreover, the absence of the *cre* cassette, the *nptII* cassette were tested by further Southern analyses.

The genetic stability of the presence of the *cordapA* gene cassette and absence of both *cre* and *nptII* gene cassettes in maize event LY038 was further confirmed by Southern blot analyses over multiple generations. Additionally, heritability of the *cordapA* gene in the GM maize was evaluated by determining segregation ratios of four generations based on Chi-square analysis.

Expression analysis using ELISA assays revealed that in LY038 grain the mean transprotein (cDHDPS) levels were about 26 µg/g dwt (dry weight), and that cDHDPS expression was predominantly in the grain tissue.

From the authors' point of view, Southern blot analyses should be amended by sequencing data in order to verify the integrity of the inserted DNA sequences (promoters, introns, coding regions, etc.). Sequence data are also needed for a proper evaluation of the occurrence of any DNA rearrangements or mutations in the flanking regions of the insertion site (at least 1000 bp should be sequenced on either side). The comparison with a negative segregant, however, does not provide reliable data for proper characterisation of the transgenic insert.

5.8.2 Comparative assessment

A phenotypic and compositional evaluation was conducted by the applicant to assess the equivalence between the GM maize event LY038 and the conventional maize. Both laboratory experiments and multi-site field tests were carried out. The field tests included, besides the GM maize, a negative segregant as isogenic control line and several conventional reference maize hybrids (USDA 2005; Health Canada 2006).

The phenotypic evaluation consisted of endpoints characterising of dormancy, germination, emergence, vegetative growth, reproductive growth, seed retention on plant, and plant interactions with disease, insect, and abiotic stressors. The compositional analysis included forage and grain of LY038 collecting several plant components including natural toxicants and significant nutrients.

The phenotypic assessment resulted in one significant result (seedling vigor rating was lower for the GM maize), and no trend across sites was found as the within-site analysis were randomly distributed among the endpoints. The detected differences were considered alone, in consideration of other observed differences, and for trends across locations. The phenotypic characteristic data showed no biologically meaningful differences (USDA 2005).

The compositional analysis used a calculated 99% tolerance interval calculated to contain, with 95% confidence, 99% of the values contained in the population of commercial maize. The 14 statistically significant differences between the GM line and the control line measured were attributed to the intentional changes in the lysine or free lysine content and associated lysine catabolites. Other observed significances were found to be within the calculated 99% tolerance interval for the population of conventional reference varieties.

The American GM plant risk assessment authorities accepted the argumentation of the applicant that no biologically meaningful phenotypic changes were associated with GM maize LY038, and that these results indicated a lack of altered weediness potential for LY038 (USDA 2005; Health Canada 2006; Canadian Food Inspection Agency 2011b).

Other risk assessment agencies came to similar conclusions: Food Standards Australia New Zealand (2006) argued the grain of LY038 corn is considered to be compositionally equivalent to that of conventional corn, and that no consistent differences in concentrations of essential amino acids other than lysine, including

methionine, threonine and isoleucine - which share a portion of the lysine biosynthetic pathway in plants - were observed.

From the authors' point of view, the comparative assessment of GM maize LY038 as evaluated by the national risk assessment agencies has clear deficits: A negative segregant was used as isogenic control line in all of the field studies conducted by the applicant. A negative segregant is a descendant of the GM plant, and therefore not suitable for establishing a proper baseline for any endpoint in state-of-the-art equivalence testing. (Current EU law and EFSA guidance define an appropriate comparator (conventional counterpart) as "*a similar food or feed produced without the help of genetic modification*" (EC 2003; EFSA 2011a)). In addition to that, the use of a 99% tolerance interval for establishing a baseline for natural variation is not to be accepted, since such approach provides little statistical power due to the large intervals. The available field trial results can be used for carrying out a statistical analysis using 95% confidence intervals instead.

5.8.3 Toxicological and allergological assessment

The safety of the transgenic cDHDPS protein expressed in LY038 maize was evaluated by USDA (2005) on the basis of:

- a history of safe exposure for the cDHDPS protein demonstrated, based on the similarity of the transprotein to naturally DHDPS proteins in food and feed,
- the safety of the donor organism (*Corynebacterium glutamicum* is not known to be human or animal pathogen),
- bioinformatics analyses showing no similarities of any eight amino acid peptide sequences derived from cDHDPS and known toxins or allergens.

Furthermore, the results of a mouse acute oral toxicity study were presented by the applicant showing that the cDHDPS protein was not acutely toxic at a dosage of 800 mg/kg and did not cause any adverse effects. So, the safety of the transgenic protein expressed in maize is based mainly on an acute toxicity study (USDA 2005).

The same test regimes were evaluated by other risk assessment agencies. These concluded as well that no significant risk to livestock and workers/by-standers was expected from exposure to the cDHDPS protein as the transprotein is neither toxic at high levels in mice nor has any similarity with known protein toxins and allergens (Food Standards Australia New Zealand 2006).

The Japanese authorities mentioned the additional evaluation of effects on wild animals of saccharopine, α-aminoadipic acid, and pipecolic acid (metabolites of the lysine catabolism), which are increased in the GM maize LY038. For this case, a feeding study (concerning broiler chicken), review of the literature and acute toxicity tests were conducted. The feeding study with broiler chicken resulted in no statistically significant differences. The acute studies in mice (saccharopine, α-aminoadipic acid) and rats (pipecolic acid) also showed no adverse effects at dosages up to 2,000 mg/kg (Japan Biosafety Clearing-House 2008).

From the authors' point of view, although the toxicological assessment included safety tests on the newly expressed protein cDHDPS and the unintended changes in concentrations of three lysine metabolites, no conclusive data in relation to the repeated human and animal consumption of GM food/feed were generated by the acute oral toxicity studies.

Another major weakness of the toxicological and allergological evaluation of GM maize LY038, as conducted by the applicant, is that potential unintended effects undiscovered during the comparative assessment were not

sufficiently addressed during the risk assessment by the applicant. Especially, whole plant toxicity studies with the whole GM maize LY038 were not performed, despite major uncertainties about the substantial equivalence of the GM line and conventional maize (see Chapter 5.8.2).

Coming to the toxicity profile of L-lysine itself, toxicity studies of different qualities, outcomes and validity exist. Thereby, a very comprehensive profile of lysine was given by Procter & Gamble rather recently (end of 2011) in the course of a GRAS notification (EAS Consulting Group 2011) (which was not denied by FDA (2012b)):

There, besides many others, some studies were mentioned which are of good quality, covering different fields of toxicological endpoints, and are suitable to be taken into consideration when estimating a tolerable daily intake of lysine:

The GRAS notification (EAS Consulting Group 2011) gives the following results:

1) In a valid subchronic toxicity study conducted in compliance with Good Laboratory Practices (GLP), the toxicological and behavioural effects of L-lysine hydrochloride in male and female Sprague-Dawley rats were evaluated. Six week-old rats (12/sex/group) were divided into four groups and were fed a diet containing 0, 1.25, 2.5 and 5% (w/w) L-lysine hydrochloride (added lysine) for 13 consecutive weeks. The average intake of lysine for these four groups in males was reported as 841.2 +29.3 mg/kg bw/d, 1677.4 ± 69.4 mg/kg bw/d, and 3356.6 ± 115.1 mg/kg bw/d and for females it was 967.9 ± 46.5 mg/kg bw/d, 1917.2 +86.5 mg/kg bw/d, and 3986.3 ± 282.9 mg/kg bw/d, respectively. The administration period was followed by a 5-week recovery period, during which time only the standard diet was provided to all animals. No treatment-related changes in clinical signs, body weights, diet consumption, water intake, ophthalmology, gross pathology, organ weights, or histology were noted in any of the groups. Among the serum chemistry and urinalysis parameters in males, a lysine-related drop in serum concentration chloride (controls, 111.9±0.7 mEq/L; 1.25% group, 109.9±1.1 mEq/L; 2.5% group, 110.3±1.5 mEq/L; 5.0% group, 109.8±1.3 mEq/L), and in females of the 5.0% concentration group (controls, 112.7±1.2 mEq/L; 5.0% group, 110.1±1.2 mEq/L) an increase in urinary excretion of chloride were noted. No other significant changes in haematology, clinical chemistry or urinalysis parameters were noted. The changes in chloride concentrations were considered by the investigators as a compensatory response to the ingested hydrochloride. Additionally, no functional, biochemical, or histological changes in renal function were noted. Histological examinations did not reveal any significant treatment-related pathology. The minor changes noted were few and not dose-related. Based on the results of this study, the investigator determined a no observed adverse effect level (NOAEL) for L-lysine monohydrochloride of 5% (~ 3670 mg/kg bodyweight (bw)/d) for both genders (male, 3360 ± 120 mg/kg/d female, 3990 + 280 mg/kg/day), the highest dose tested.

2) For examination of the impact of supplemental lysine on pregnancy course and outcome in female Sprague-Dawley rats: From day 0 until day 20 of pregnancy, rats were fed a complete 20% casein diet containing lysine at 50, 100, or 500% excess over controls. The addition of lysine was 0.78, 1.56, and 7.79 mg/100 mg of the diet. One group of rats received a control diet ad libitum and each treatment group had a matched pair-fed group receiving a control diet. The control diet content of lysine was 7.78 mg/100 mg. Based on daily consumption of feed and body weight, the resulting dose of lysine was estimated as 140, 280, or 1400 mg/day (1120, 2240, or 11200 mg/kg bw/day), respectively. The groups receiving 50 and 100% excess lysine showed no significant differences from controls in maternal weight gain or fetal condition. Compared to control, the treatment group receiving a 500% excess of lysine had lower maternal weight gain and smaller fetuses. The investigators concluded that variable levels of lysine supplementation did not cause recognisable fetal malformations. However, diets highly supplemented with lysine may affect pregnancy through a decrease in maternal weight gain and fetal size. The 500% excess lysine relates to human supplementation with lysine of

approximately 35000 mg/d. The results of this study suggest that feeding of supplemental lysine in diet to rats during pregnancy had no adverse effects on pregnancy course and outcome at levels up to 2240 mg/kg bw.

Even though the GMO product is only intended for use as or in feeding-stuff, because maize constitutes an important, in many regions of the world even staple food, and because such maize theoretically cannot be excluded from becoming or adding to human diet (as laid down in detail in chapter 2.5), human consumption patterns have to be taken into consideration and are evaluated in the following.

Tomé and Bos (2007) describe the lysine intake in diets of Western populations to be in the range of 40 - 180 mg/kg bw/d. For example, the lysine concentration in conventional maize ranges between 2.5 mg/g and 2.8 mg/g (Monsanto Company 2004).

The above-mentioned dossier (EAS Consulting Group 2011) describes a mean usual daily intake of lysine for all individuals from foods and supplements of 5270 mg/day, corresponding to about 130 mg/kg bw/d for children and about 66 mg/kg bw/d for men. Related to the 60 kg standard person this would mean about 88 mg/kg bw/d, fitting into the lower third of the range described by Tomé and Bos (2007).

Therefore, a roughly 30-fold margin of safety can be calculated for children considering the outcome of the subchronic toxicity study, and a roughly 60-fold safety margin for men. Taking into account the teratogenicity study, an approximately 25-fold safety margin can be calculated. These factors seem to be rather low as against ordinary ("state of the art") safety factors of at least 100 with usual toxicological evaluations. But one has to keep in mind that L-lysine is an essential amino acid and large overdoses may lead rather to imbalances of the amino acid profile than to toxicity itself. But nevertheless, food supplements with high doses of lysine have to be taken with caution, since it bears risk factors and side effects which cannot be ignored.

Regarding allergic reactions, an excessive intake of lysine can have such effects. Although the allergies differ from person to person, patients will often experience swellings around the face, lips and tongue. It also causes blocking of the throat, and difficulties in breathing (Med-Health.net 2014). Even though such effects have been reported only with very high doses, allergy coming from Lysine is principally possible and has to be noticed.

Specifically for the situation regarding the human consumption of maize in Austria (for details see chapter 2.5) the following conclusions can be drawn:

Compared to the daily intake of lysine from all sources the contribution of the GM maize even with the maximum intake scenario and calculating the highest consumption is very low (2% maximum), shifting the safety margin only negligibly.

In conclusion, no oversupply of lysine can be expected due to the (not intended but theoretically possible) human consumption of products derived from GM maize LY038.

5.8.4 Risks associated with pleiotropic effects

As has been said for nutritionally altered GM plants, targeting a plant's metabolic pathway by genetic modification can lead to unintended effects due to the complexity of underlying mechanisms and unknown regulatory or interactions of molecules.

In the GM maize LY038, unintended changes in relation to lysine catabolism metabolites were observed concerning an increase in the lysine-related catabolites saccharopine, α-aminoadipic acid and pipecolic acid. In the comparative assessment it was found, however, that the levels fell within the ranges measured for commercial maize varieties.

The increase in saccharopine and α-aminoadipic acid was said to be expected, although not intended. This effect was accompanied by significant differences in the levels of other amino acids (glutamic acid, histidine, isoleucine, and phenylalanine) compared to the control line. This observation is in accordance with the results of Zhu and Galili 2003, who associated the lysine overaccumulation with altered levels of several amino acids that are metabolically related to it (e.g. glutamic acid).

Pipecolic acid is a nonprotein amino acid and lysine catabolite that is widely distributed in plants and plays a substantial role in systemic plant immunity (Návarová et al. 2012). The mean pipecolic level in GM maize LY038 was 28.72 µg/g (dw), but in the control plant is was only 14.96 µg/g (dw).

The Japanese authorities requested a broiler chicken feeding study and acute toxicity tests with saccharopine and α-aminoadipic acid. A broiler chicken feeding study, however, is not a suitable experiment for providing information on the toxicity of substances for humans. Also, acute test are not relevant for evaluation of the food safety of substances.

No significant differences between lysine enhanced GM maize and its conventional counterpart were observed, other than the abovementioned.

From the authors' point of view, 90-day toxicity studies should be performed with the whole GM maize and also adequate (repeated dose) toxicity studies with any protein/substance, that has not yet been appropriately biochemically and toxicologically characterised. This concerns both the newly expressed cDHDPS protein(s) and any metabolite that shows significant changes (saccharopine).

5.8.5 Exposure assessment

The GM plant LY038 is a transgenic maize with an increased lysine concentration. Its intended use is as ingredient in poultry and possibly swine diets. Lysine is an essential amino acid because it cannot be synthesised by mammals. Additionally, it is a limited protein in all cereals. Therefore, this GM maize is blended with feed to improve the development and growing of the animals. This GM plant is not intended to be used for human consumption (Monsanto Company 2004, Tomé and Bos 2007). An exposure assessment was performed as precautionary principle, because the GM plant and derived products may appear in food by mistake.

It is described that in LYO38, the expected total lysine would range from 3.5 - 5.3 mg/g on a dry weight (dw) basis (Monsanto Company 2004). For the exposure assessment the highest concentration (5.3 mg/g) was used in the calculations. A factor, relating to the lysine concentration in fresh weight (fw) products has to be included.

The exposure assessment refers to the following products: steamed maize, canned maize, fresh maize, cornflakes, maize flour, maize starch, maize bread, maize oil. The factor of fresh weight is determined by the database BLS ("Federal Food Stuff Key"):

For example, the water amount of cornflakes is about 4.5%. The fresh weight/dry weight conversion factors is 1 - 0.045 = 0.955 g dw/ g fw. The total lysine concentration is calculated:
5.3 mg/g x 0.955 g dw/g fw = 5.0615 mg/g fw. Then the calculated total lysine concentration based on fresh weight is multiplied by the intake of cornflakes.

Such calculation was performed with each of the maize products mentioned before. The maize products intake of men, women and children is based on the food consumption database of the "Austrian Nutrition Report

2008" (Elmadfa et al. 2009). The lysine intake was estimated based on the total intake, and as well based on the bodyweight of men (81.5 kg), women (63.6 kg) and children (39.7 kg) (see Table 25).

Table 25 illustrates the intake of lysine from consumption of maize products for the Austrian population. In the maximum intake scenario it is estimated that 100% of the consumed maize belongs to LY038. For example, the 100% GM total intake of lysine in men is 38.13 mg/d of the 95th percentile and in comparison children have an intake of 115.44 mg/d. Men consume a bit more of maize products than children, but it must be explained, that for each maize product an individual factor for estimating the lysine content in fresh weight, is involved. For example children are eating more cornflakes than men and so a different lysine intake due to GM cornflakes consumption was calculated. Different intakes of lysine are caused by different eating habits in the populations, whereas the total intake of maize products is similar.

Table 25: Intake of lysine from maize products (maximum intake scenario 100% GM) of men, women and children in Austria in mg/d and based on mg/kg bodyweight (bw)/d

GM maize LY038	Men		Women		Children	
	Collective		Collective		Collective	
	Mean	P95	Mean	P95	Mean	P95
maize products intake (g/d)	5.38	33	5.74	33	9.5	31.7
100% GM total Intake of lysine (mg/d)	13.03	38.13	12.95	38.13	35.17	115.44
100% GM total Intake of lysine (mg/kg bw/d)	0.16	0.47	0.20	0.60	0.88	2.90

P95 = 95th percentile; bw = body weight

Table 26: Protein and lysine requirements of infants, children, adolescents, and adults (Tomé and Bos 2007)

Age (y)	Protein (g/kg bw/d)		Lysine[1]
	Maintenance	Growth	(mg/kg bw/d)
0.5	0.686	0.46	64
1 - 4	0.686	0.19	45
4 - 10	0.686	0.06	35
10 - 14	0.686	0.07	36
14 - 18	0.686	0.04	34
>18	0.66	–	30

[1] Sum of the lysine requirement for maintenance (maintenance protein x the adults scoring pattern) and growth (tissue deposition for a 58% dietary efficiency of utilisation x the AA tissue pattern).

The results of the estimated intake of lysine from GM maize LY038 based on the bodyweight (bw) of the Austrian population is demonstrated in Table 25. The requirements regarding lysine intake for infants to adults are shown in Table 26. The result of the maximum intake scenario can be compared with the requirements: Even in the case of unintended mixing of food with GM maize LY038, no excessive intake of lysine due to the consumption of maize products is to be expected.

5.9 GM plants with increased erucic acid content

Erucic acid is a C:22 ω-9-fatty acid with a single double bond. It usually constitutes about 30 - 60% of the total fatty acids of rapeseed, mustard seed and wallflower seed and also occurs in marine animal oils. Selective breeding in association with safety concerns of erucic acid has led to oilseed rape varieties containing now, at least, less than 2% of this fatty acid (as % total fatty acids) (Food Standards Australia New Zealand 2003), which is the maximum value accepted in the European Union for rapeseed 00 (double zero) used in the food industry (FAO/WHO 1999; Schumann et al. 2006).

The enhancement of erucic acid in rapeseed varieties is mainly founded on interests of the oleochemical industry for which it is an important renewable raw material. For instance, it is used to produce erucamide, an important slipping agent used in the production of polyethylene and propylene films. Another application is in synthesis of nylons and as raw material in the lubricant and emollient industries. Erucic acid is now mainly derived from, conventionally breed, high-erucic acid rapeseed (HEAR), and the contents are in the range of 45% - 55% of total fatty acid. A second important plant source for erucic acid is *Crambe abyssinica* (Katavic et al. 2000; Nath et al. 2009; Vanhercke et al. 2013).

GM oilseed rape with increased erucic acid content is developed by use of a combination of two transgenic approaches: the insertion and over expression of a *fae1* elongase transgene from *Arabidopsis* combined with the expression of a *Ld-LPAAT* (lysophosphatidic acid acyltransferase) gene from *Limnanthes douglasii* (Nath et al. 2009).

The elongase encoded by the *fae1* gene katalyses the production of erucoyl-CoA, and is needed because of the low erucoyl-CoA pool available for the biosynthesis of the trierucin (trierucoylglycerol) (Puyaubert et al. 2005). This triglyceride is a plant storage lipid and is used as an indicator for erucic acid contents in high erucic acid plants.

The sn-2 acyltransferase of oilseed rape (*B. napus*) does not accept erucoyl-CoA as a substrate, and therefore the insertion of erucic acid into the sn-2 glycerol position is the limiting factor in the production of erucic acid. The transgenic lysophosphatidic acid acyltransferase from *Limnanthes* species enables the insertion of erucic acid into the sn-2 glycerol position (Nath et al. 2009).

A combination of this two genetic modifications of oilseed rape was performed in a breakthrough study by Nath et al. (2009) using a high erucic acid winter oilseed rape breeding line (BGRV2) with an erucic acid content of 52% in the seed oil for the transformation experiments. After transformation, the transgenic line (generation F_0) was crossed with another oilseed rape variety (6575-1 HELP) which contained about 50% erucic acid. Plants were grown in greenhouses and F2 and F3 generations examined for an increased erucic acid content. In the F2 generation, the concentration was found to be raised by 8.9% (up to 62.5% erucic acid), and in the F3 generation even levels up to 72% erucic acid were detected with a simultaneous decrease of polyunsaturated fatty acids. The achievement of levels around 72% erucic acid represents a milestone in the breeding of high erucic acid oilseed rape (Nath et al. 2009).

5.9.1 Molecular characterisation

5.9.1.1 Molecular characterisation of rapeseed containing transgenes interfering with fatty acid metabolism

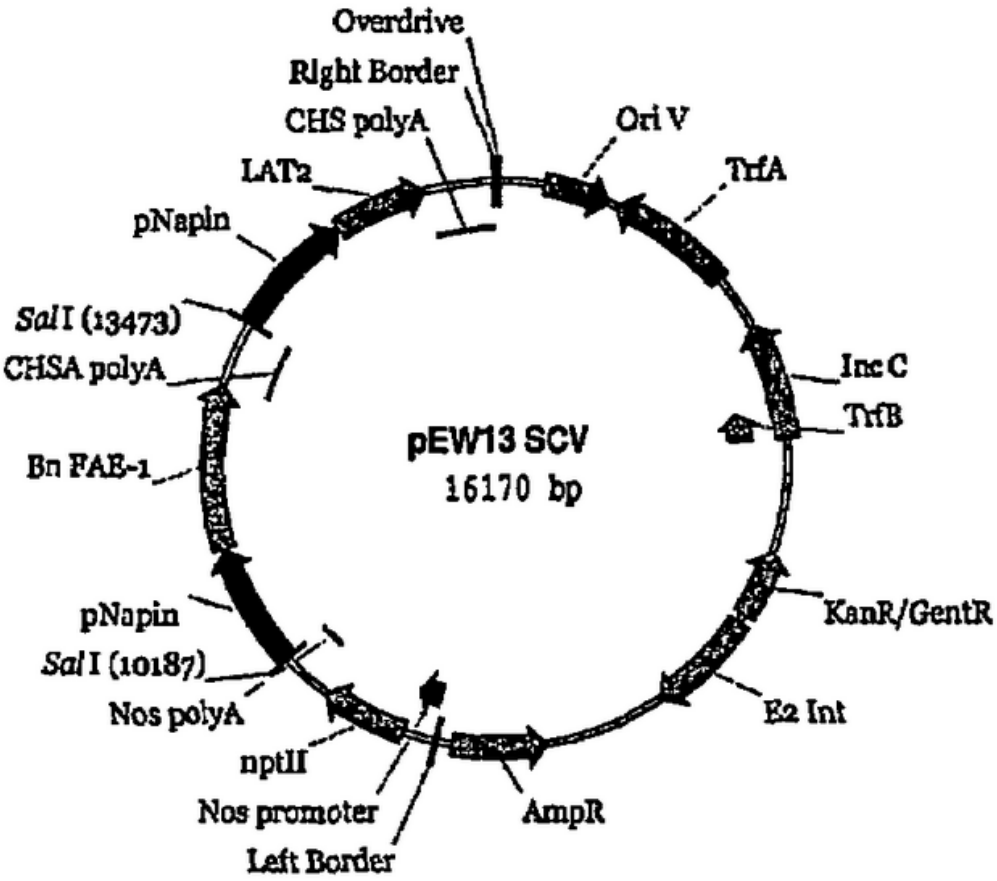
In the following section, transgenic high erucic acid rapeseed plants with an expression cassette containing the rapeseed fatty acid elongase *fae1* and the lysophosphatidic acid acyltransferase gene *Ld-LPAAT* as presented in Nath et al. (2009) are described.

Construction and transformation experiments with the transgenic insert

To compensate for insufficient fatty acid elongation and lack of insertion of erucic acid into the central sn-2 position of the triacylglycerol backbone in conventional rapeseed lines the rapeseed fatty acid elongase gene *fae1* (coding for a ß-ketoacyl-CoA synthase) and the gene for the lysophosphatidic acid acyltransferase (*Ld-LPAAT*) from *Limnanthes douglasii* were cloned into the binary shuttle vectors pEW13 (containing variant 1 of *fae1*; see Figure 17) or pEW14 (containing variant 2 of *fae1*). The addition of *fae1* leads to overexpression of the elongase, *Ld-LPAAT* facilitates the insertion of erucic acid in the sn-2 glycerol position.

Agrobacterium tumefaciens strain C58 pMP90 carrying either the binary plasmid pEW13 or pEW14 (see Figure 17) was used to transform the conventional high erucic acid winter oilseed rape breeding line BGRV2. 10 transgenic plants (T1) carrying plasmid pEW13 and 18 transgenic plants carrying plasmid pEW14 were regenerated, and the oil composition was analysed after several steps of self-pollination. Erucic acid content in these lines varied between less than 30 and about 65%. Line 361.2B, transformed with pEW14 (*fae1-2*), was selected as it had the highest erucic acid content in T3 seeds (Wilmer et al. 2008). To increase the erucic acid content further, T4 seeds of 361.2B were crossed with the conventional high erucic acid winter rapeseed line 6575-1 HELP.

Figure 17: pEW13 binary vector used for transformation of oilseed rape breeding line BGRV2 (from Wilmer et al. 2008)



RB, LB	right and left borders of the T-DNA
Nos	nos promoter
nptII	kanamycin resistance gene
nos polyA	nos terminator
pNapin	Brassica napus napin storage protein promoter region
Bn FAE-1	ß-ketoacyl-CoA synthase variant 1 gene
CHSA polyA	chalcone synthase gene terminator
LAT2	lysophosphatidic acid acyltransferase gene (Ld-LPAAT)

Expression and stability of the high erucic acid trait

The presence of the correct inserts was established by gene-specific *fae1-2* and *Ld-LPAAT* PCR. Segregation analysis of the F2 generation (15:1) indicated the presence of two transgene copies in the transgenic parent 361.2B.

The stability of the high erucic acid trait was checked by gas liquid chromatography (GLC) analysis of trierucin content (test of functionality for the *Ld-LPAAT* gene*)* and the determination of the fatty acids profile of F2 and F3 plants by GLC. The frequency distribution for the erucic acid content of the F2 population showed a large and continuous variation as expected for a polygenic inherited trait. Erucic acid content varied from 44 to 72%

in the F2 generation and from 50 to 72%, showing a mean of 64.8%, in F3. F2 plants with about 70% erucic acid content in the seed oil were confirmed in the F3 generation. The increase from 50% (in currently available conventional rapeseed cultivars) to 72% erucic acid in transgenic crossings is considered as a milestone improvement (Nath et al. 2009).

There was no explicit testing for genetic stability. Phenotypic stability was analysed for two generations in the greenhouse and was attributed as relatively stable because the parental lines had similar erucic acid values at two different seasons by Nath et al. (2009). For a proper risk assessment the data for at least five generations must be available (EFSA 2011a).

From the authors' point of view, data are necessary for covering systematically molecular characterisation, genetic stability and gene expression aspects of the transgenic rapeseed plants. Additionally, the transgenic construct contains an antibiotic resistance marker gene (*nptII*), which should be avoided, according to current EU regulation (EC 2013a).

5.9.2 Comparative assessment

The synthesis of long chain monounsaturated fatty acids in plants is located in the cytosol and catalysed by a membrane-bound fatty acid elongation (FAE) complex on the endoplasmic reticulum. In general, a comparative assessment of an erucic acid high GM oilseed rape should include the parameters mentioned in the respective OECD consensus document (OECD 2001).

It is, however, important to ensure that all potential fatty acids which may be affected by the genetic modification of the FAE complex are included in the comparative analysis. The product of the elongation process depends on the preferably used precursor. If the elongase utilises 18:1 Δ9, the resulting product is 20:1 Δ11 (eicosenic acid), but if the elongase utilises 20:1 Δ11, than the resulting product would be 22:1 Δ13 (erucic acid). The preferred precursor depends on different factors as e.g. the plant species (Katavic et al. 2002). This could result in a high variation for metabolised fatty acids.

Comparative studies were presented by Nath et al. (2009) in their study with transgenic oilseed rape containing levels of up to 72% erucic acid (as % total fatty acids) in the seeds. The comparisons were made between the transgenic F2 generation and the transgenic parental lines, which is not the same procedure as defined by current regulation and guidances for evaluation of substantial equivalence of GMO plants and derived food and feed (EFSA 2011a; EC 2013a). However, the comparisons provided an insight into some side-effects due to the enhancement of the erucic acid content in a rapeseed plant: The comparisons concerned total fat content, protein content and several fatty acids, and the F2 lines showed high variations as compared to the two parental transgenic lines (361.2B and high erucic acid 6575-1 HELP) since the protein content was higher and the oil content lower. The ranges of the F2 lines varied from 6 - 26% for PUFA, from 5 - 26% for oleic acid and from 19 - 33% for protein content. As expected, the erucic acid content was increased by 8.9% as compared to the non-GM high erucic acid parental line.

Six selected F3 lines were also compared with the parental line 361.2B with the additional application of the Fisher's least significant differences procedure as a statistical tool for significance tests at P = 0.05. The most important results were that the six F3 lines showed significantly lower concentrations in saturated acids (stearic acid, palmitic acid) than the parental lines. As expected, all of the six F3 lines had higher erucic acid contents than the parental lines.

Another evaluation by Nath et al. (2009) was to calculate correlations between the (enhanced) erucic acid content in the F2 lines using a plant breeding statistical program. A negative correlation between erucic acid and protein, SFA, oleic acid, PUFA and eicosenoic acid content was shown, but positive correlations between

erucic acid content and oil and trierucin content were observed. For instance, a reduction of 10% in PUFA was found to be correlated with an increase of 6.5% erucic acid.

From the authors' point of view, as these results indicate unintended changes in the protein profile and the fatty acid metabolism due to the genetic modification, a comprehensive comparative analysis, according to standards laid down in current EU regulation (EC 2013a), needs to be performed addressing the abovementioned issues. This analysis could be the starting point for further evaluations.

5.9.3 Toxicological and allergological assessment

Toxicological data on erucic acid show that an association between the dietary intake of this acid and myocardial lipidosis exists in a number of species (Kramer et al. 1992; Food Standards Australia New Zealand 2003):

Myocardial lipidosis - a disease provoked by an abnormal accumulation of lipids in the cardiac muscle of the heart - is associated with doses of erucic acid at 1500 mg/kg bw/day in rats and 900 mg/kg bw/day in nursling pigs. The lower incidence dose in nursling pigs might be due to the immature myocardium or liver is less able to oxidise long-chain fatty acids.

Different study results, which are described in detail by Food Standards Australia New Zealand (2003), have also shown that in pigs and monkeys no other adverse findings can be associated with erucic acid consumption, other than myocardial lipidosis. Rats, however, typically also develop myocardial necrosis followed by fibrosis, at erucic acid doses of 6600 mg/kg bw/day. Rat feeding studies have further demonstrated an association between dietary erucic acid and heart lesions.

So far, however, there is no evidence that dietary erucic acid can be correlated to either of the abovementioned effects (myocardial lipidosis, heart lesions, etc.) in humans. Epidemiological studies exist indicating that erucic acid may occur in human heart muscle in geographic areas where vegetable oils containing erucic acid are consumed, but no clear association has been established (Food Standards Australia New Zealand 2003).

Using the data of the animal feeding studies and an uncertainty factor of 100 (10 for extrapolation to humans, 10 for variation within humans) a tolerable level of human exposure was calculated to be 7.5 mg erucic acid/kg bw/day.

Coming to a risk evaluation, the exposure of the Austrian population was compared with a PTDI-value of 7.5 mg/kg bw/day. In the maximum intake scenario, all population groups are exceeding this value multiple times. No exceedances of erucic acid are observed in the minimum intake scenario.

However, the risks are negligible, since the erucic acid content for oilseed rape and derived products intended for human consumption and for use in animal feed is regulated by Codex Alimentarius (FAO/WHO 1999) stating, "*Low-erucic acid rapeseed oil must not contain more than 2% erucic acid (as % of total fatty acids).*" Moreover, the erucic acid content of EU food products is regulated in Council Directive 76/621/EEC, "*In oils, fats and mixtures thereof which are intended as such for human consumption the level of erucic acid calculated on the total level of fatty acids in the fat component, may not be greater than 5%*" (EEC 1976).

In conclusion, it has to be noted that the intended use of this GM rapeseed is just of industrial purpose and not for human consumption, and that the GM rapeseed with high erucic acid is subject to the same regulations and restrictions for use as any conventional non-GM high erucic acid oilseed rape varieties. Aspects in relation to the unintended commingling of the food chain are mentioned in Chapter 5.9.5.2.

In addition, it is important to note that other substances than the erucic acid produced by the GM plant may give rise to potential risk for consumers as well, e.g. the newly expressed proteins. For demonstrating the safety for humans and animals a thorough evaluation of these novel proteins has to be performed starting with a biochemical characterisation (structure, function, enzymatic activity, etc.) and including bioinformatics, digestibility and heat stability tests. Particularly, any allergenic potential of the novel proteins has to be thoroughly evaluated, since occupational allergies were reported in connection with oilseed rape flour among farmers (Alvarez et al. 2001). Immunological assays could be used to evaluate the overall allergenicity of the high erucic acid GM oilseed rape as compared to the non-GM conventional counterpart. In this respect, sensitisation tests including skin tests or specific IgE measurements using serum of allergic individuals provide valuable information.

A subchronic 90-day toxicity study in rodents with the whole GM oilseed rape is not indicated because this GM plant is not intended for feed and food uses. Furthermore, the high erucic acid content would make a whole GM plant toxicity study unfeasible.

5.9.4 Risks associated with pleiotropic effects

In the comparison studies with GM high erucic acid oilseed rape, effects other than the intended increase of the erucic acid content were observed (Nath et al. 2009). Such observation is not surprising, since genetic modification processes were shown to potentially lead to unintended effects, one of which is a changed level of metabolites (Filipecki and Malepszy 2006). Especially, genetic engineering for a qualitative or quantitative change in important plant compounds (e.g. proteins, fatty acids) may lead to modifications in plant metabolic systems and plant physiology (ADAS 2013).

The unintended effects, as observed in high erucic acid GM oilseed rape by Nath et al. (2009), primarily concerned the levels of stearic acid and palmitic acid, which were significantly lower in the GM plant lines (F3 generation). The mean values (measured as % of total fatty acids) for these two saturated fatty acids in the different GM lines tested ranged from 1.9 - 2.3% as compared to the non-GM parental lines (2.9 - 3.3%).

In contrast, the F2 generation of GM plants revealed significant differences in total protein level, and also the oil content was changed: the protein mean was 24.8% compared to about 24% for the non-GM parental lines, and the oil content was 47.7% compared to about 49.7% for the non-GM parental lines (Nath et al. 2009). This result indicated a metabolic shift towards fatty acid synthesis at the expense of protein. An effect for lower saturated fatty acid levels, however, was not seen in the F2 generation.

No other effects have been reported for GM oilseed rape with high erucic acid contents, and also no further data on field trial evaluations providing detailed analysis of compositional and phenotypic differences as compared to conventional oilseed rape are available.

From the authors' point of view, a comprehensive evaluation should be carried out including a comparative assessment and a toxicological risk assessment with respect to any novel proteins produced by the GM oilseed rape. A subchronic 90-day toxicity study in rodents with the whole GM oilseed rape, however, is not indicated. (Please see also Chapter 5.9.3.)

5.9.5 Exposure assessment and unintended commingling

5.9.5.1 Exposure assessment of GM oilseed rape with high erucic acid content

Rapeseed oil with a high amount of erucic acid (HEAR) is of big interest for the oleochemical industry. Erucic acid and its derivatives are used in plastic film, nylon, lubricant, cosmetic and emollient industries. Rapeseed

oil is a renewable raw material and the increased erucic acid content would reduce processing costs. This GM rapeseed oil expresses 72% erucic acid. The use of this oil is only for industrial processes, because high levels of erucic acid in cooking and salad oil extracted from rapeseed are associated with health problems. An exposure assessment was performed as precautionary principle, because of the potential unintended commingling of food (e.g. oil) with GM material.

In most parts of the world, oilseed rape with low erucic acid content and low glucosinolate content in seeds are predominantly grown, because of the unwanted side effects of this C:22 fatty acid (Nath et al. 2009). As already described, the amount of erucic acid is 72% of rapeseed oil and based on the BLS ("Federal Food Stuff Key"), the amount based on total fat is determined. The total fat of rapeseed oil of a brand from Austria is about 99.9%. For the exposure assessment, the consumption data of vegetable oils and margarine are used from the "Austrian Nutrition Report 2008" of men, women and children. These two products are selected for the calculations, because rapeseed oil is a common used vegetable oil and as well applied in the margarine production. The calculation is performed by multiplying the erucic acid concentration by the intake of rapeseed oil products. To refer it to the bodyweight of men (81.5 kg), women (63.6 kg) and children (39.7 kg), the result is divided by the operating figures of the body weight (Elmadfa et al. 2009).

Table 27 demonstrates the intake of erucic acid derived from vegetable oil and margarine based on the total intake and on the bodyweight. The maximum intake scenario describes the consumption of rapeseed oil products which derive 100% from GM rapeseed. The minimum intake scenario is based on the assumption that 0.9% of the consumed rapeseed oil products are derived from GM rapeseed. The amount of 0.9% may be in a non-GM rapeseed product because of unavoidable contamination (EC 2003) (see explanations in Chapter 5.1 "Method used for the exposure assessment").

Table 27: Intake of erucic acid from vegetable oil and margarine (maximum intake scenario 100% GM and minimum intake 0.9% GM) of men, women and children in Austria in mg/d and based on mg/kg bw/d

GM rapeseed	Men		Women		Children	
	Collective		Collective		Collective	
	Mean	P95	Mean	P95	Mean	P95
Intake of vegetable oil & margarine (g/d)	4.00	17.00	2.90	20.10	1.40	6.70
100% GM erucic acid (mg/d)	2879.80	12239.14	2087.85	14470.99	1007.93	4823.66
0.9% GM erucic acid (mg/d)	25.92	110.15	18.79	130.24	9.07	43.41
100% GM erucic acid (mg/kg bw/d)	35.34	150.21	32.85	227.66	25.36	121.37
0.9% GM erucic acid (mg/kg bw/d)	0.32	1.35	0.30	2.05	0.23	1.09

bw = bodyweight; P95 = 95th percentile

Table 28 demonstrates an NOEL (no observed effect level) and PTDI (provisional tolerable daily intake) of erucic acid based on a toxicological review and risk assessment of erucic acid in food published by the Food Standards Australia New Zealand (2003).

The exposure of the Austrian population was compared with the PTDI-value of 7.5 mg/kg bw/day.

Table 28: NOEL and PTDI of erucic acid (Food Standards Australia New Zealand 2003)

NOEL	750 mg/kg bw/d
PTDI	7.5 mg/kg bw/d

NOEL = no observed effective level; PTDI = provisional tolerable intake; bw = body weight

In the maximum intake scenario, all population groups are exceeding the PTDI of 7.5 mg/kg bw/d, for multiple times. No exceedances of erucic acid are observed in the minimum intake scenario.

In conclusion, and as mentioned in Chapter 5.9.3, according to EU law (Council Directive 76/621/EEC), oils, fats and mixtures thereof which are intended as such for human consumption the erucic acid content must not be greater than 5% (EEC 1976). For example, an Austrian rapeseed oil from the BLS database ("Federal Food Stuff Key") contains about 0.22% erucic acid.

5.9.5.2 Unintended commingling with GM oilseed rape with high erucic acid content

The avoidance of unintended commingling of food and feed material with GM oilseed rape producing high levels of erucic acid is important for the safety of humans and animals. Potential hazards and risks posed by the unintended mixing of low-erucic acid oilseed rape cultivars developed for food and feed uses with high erucic acid GM cultivars are described in an evaluation study by Turley et al. (2011) and arise from:

- pollen flow to neighbouring conventional plant seed cultivars,
- seed remaining in soil that may produce viable plants which could contaminate conventional oilseed rape cultivars planted in subsequent seasons,
- the cultivation of non-approved high erucic acid varieties,
- contamination of conventional oilseed rape cultivars during storage and transport operations,
- contamination of conventional oilseed rape cultivars during processing,
- entering of high erucic acid oilseed rape meal by-products in the animal feed chain.

It should be especially noted, that import of seeds or cultivation of GM oilseed rape could lead to unintended emergence and establishment of feral GM oilseed rape populations outside cultivation areas. Such feral oilseed rape populations are likely to persist and contribute to the spread of transgenes. To avoid GM contamination of non-GM oilseed rape fields, efficient management systems need to be established which are complex, expensive and require heavy workload (Pascher 2012). Therefore, it is questionable whether marketing and cultivation of high erucic acid GM oilseed rape in the European Union would make economic or ecological sense.

5.10 GM plants with improved yield

The risk assessment of GM plants has to be conducted on a case by case basis taking into consideration the intended effect, the underlying genetic engineering method or approach, and the likelihood for unintended modifications at the gene regulatory, gene expression and metabolite level of the plant. It must be taken into account that many different ways of genetic modification may lead to high-yielding plants, and the risk assessment has to be adjusted accordingly. The basis requirements are laid down by current regulations and guidances (EFSA 2011a; EC 2013a) and need to be supplemented dependent on the characteristics of the GMO event (e.g. if no conventional counterpart is available due to substantial modifications).

Improved yield is an important breeding goal of modern agriculture and plant production. A number of different strategies have been developed to raise yield potential in plants. The grain yield that a plant can produce is dependent on the following parameters (Hüner et al. 2014):

- Amount of incident solar radiation available over the growing season of a plant,
- Efficiency of the photosynthetic pigments to intercept photosynthetic active radiation,
- Ratio of the biomass energy produced to the radiative energy intercepted by the canopy,
- Translocation of photosynthates to sinks,
- Amount of total biomass energy partitioned into seed production per unit ground, known as harvest index (HI).

According to current knowledge, energy partitioning efficiency and light interception efficiency have approached the upper limit. Therefore, a yield increase can only be achieved by an increase in the energy conversion efficiency into biomass (Zhu et al. 2010).

Different approaches have been proposed to address the energy conversion efficiency in plants: development of dwarf or semi-dwarf phenotypes, increasing photosynthetic capacity, optimising partitioning to grain, marker-based breeding (Reynolds et al. 2011; Hüner et al. 2014).

Dwarfism is one of the valuable traits in crop breeding in connection with increased yield, and semi-dwarfism transgenes are known to be suitable for modification of characteristics of plant development due to controlling of growth and physiological processes. Semi-dwarfism or dwarfism of plants can be induced by manipulation of the interactions between phytohormones leading to changes in e.g. root growth, lodging resistance and morphological diversity, and in particular to increased yield (Elias et al. 2012).

One target of genetic engineering associated with improved yield are the phytohormones gibberellins, which are endogenous plant hormones influencing characteristics like seed germination, leaf expansion, shoot growth, cell division, flower induction, and fruit development (Elias et al. 2012).

Another promising target associated with dwarf phenotypes and increased yields are the brassinosteroids, another class of plant hormones that is known to be involved in cell and stem elongation. Besides the development of dwarf phenotypes by designing loss-of-function mutants, also the co-suppression of a rice *OsBRI1* gene led to brassinosteroid insensitive plants. This approach was undertaken as an attempt for increasing the grain yield in transgenic rice. The experiments with the co-suppression system revealed that this method could be a feasible target for producing high-yielding rice cultivars (Morinaka et al. 2006).

Due to the high number of different approaches for the improvement of yield in plants, the following evaluation and discussion in relation to the risk assessment of high-yielding GM plants focusses on the experiments of Morinaka et al. (2006). This high quality study describes a standard breeding approach which resulted in the production of nine knock-down mutants (lines d61-1 to d61-9) and a genetic engineering approach (co-suppression strategy to repress endogenous *OsBRI1* expression using a constitutively expressed truncated sense *OsBRI1* cDNA construct (lines BKD11, BKD22)). The co-suppression strategy shows a successful way for the development of a GM rice variety with an increased yield and contains a number of relevant information that can be used for discussing the risk assessment.

5.10.1 Molecular characterisation

5.10.1.1 Molecular characterisation of transgenic rice with reduced OsBRI1 expression

In the following section, transgenic rice plants containing a partial *OsBRI1* gene which resulted in a reduction of *OsBRI1* expression are described as presented in Morinaka et al. (2006).

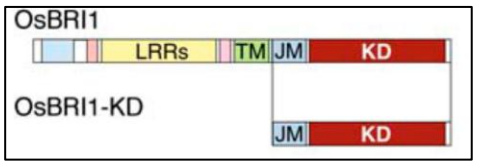
The naturally occurring *Oryza sativa* dwarf mutant d61-7 shows semi-dwarf stature and erect leaves which provides better stability against wind and rain, improve light capture for photosynthesis and nitrogen use by grain and lead to a higher leaf area index (one-side leaf area per unit of land area) in dense plantings, all of which increase yield. This phenotype is caused by brassinosteroid (BR) insensitivity caused by a loss of function of the *O. sativa OsBRI1* gene which codes for a Leu-rich-repeat receptor-like kinase that functions as a BR receptor.

However, the grain size of the naturally occurring mutant d61-7 is insufficient and counters any increase in grain yield. Therefore, additional attenuated phenotypes of mutant d61-7 were generated by transgenic OsBRI1 knock-down mutants (for details see below).

Construction and transformation experiments with the transgenic insert

Direct knock-down of *OsBRI1* by antisense *OsBRI1* cDNA showed more severe or similar phenotypes compared to the naturally occurring mutant d61-7, although the strategy was aimed at generating a milder phenotype. To finally achieve that goal a constitutively expressed truncated sense *OsBRI1* cDNA construct (OsBRI1-KD; see Figure 18) under the control of the rice actin promoter was designed (co-suppression strategy).

Figure 18: Schematic representation of the native OsBRI1 and truncated OsBRI1 (OsBRI1-KD) proteins (Morinaka et al. 2006)



LRR: lysine repeat region
TM: transmembrane region
JM: iuxtamembrane region
KD: kinase domain

To generate an OsBRI1-KD construct, a partial *OsBRI1* cDNA containing the juxtamembrane region, kinase domain, and C-terminal region was amplified by PCR and inserted between the rice actin promoter and the gene for the nopaline synthase polyadenylation signal of the hygromycin-resistant binary vector pAct-Hm2. This vector was modified from pBI-H1 (Ohta et al. 1990). *Agrobacterium*-mediated transformation of rice *Oryza sativa* L. cv Taichung 65 was performed as described by Hiei et al. (1994).

Two lines of the T1 generation were selected for further analysis because their phenotype resembled that of wild type except for the erect leaves. Both lines carried a single copy of the introduced *OsBRI1-KD* gene (Morinaka et al. 2006).

Expression and stability

To determine the accumulation level of *OsBRI1* transcripts in transgenic plants, total RNAs were prepared from whole seedlings 10 d after germination, and single-strand cDNAs were synthesised by using a realtime RT-PCR with primers specific for the extracellular domain of OsBRI1. The expression level was normalised against the values obtained for histone H3, which was used as an internal reference gene. T5 transformants were planted in pots, grown in a glasshouse environment and used for analyses (Morinaka et al. 2006).

BKD11 and BKD22 plants from the T5 generation produced erect leaves but did not show a semi-dwarf or dwarf phenotype. The gross morphology of BKD11 and BKD22 was indistinguishable from that of the wild type control. Endogenous *OsBRI1* expression was well, but not completely, suppressed. This indicated that partial suppression of OsBRI1 function would induce an erect-leaf phenotype. The grain yield of BKD11 could be calculated as 35% larger than that of wild type at high density (BKD11, 12.29 t ha^{-1}; wild type, 9.13 t ha^{-1}). Similarly, the grain yield of BKD22 was approximately 26% higher, indicating that the combination of erect-leaf plants with dense planting can increase grain production without extra fertiliser application (Morinaka et al. 2006).

There was no explicit testing for genetic or phenotypic stability. Plants from the T5 generation were used for analyses. For a proper risk assessment the data for at least five generations must be available (EFSA 2011a). There are no data available covering systematically molecular characterisation, genetic stability and gene

expression aspects of the transgenic rice plants. Additionally, the transgenic construct contains an antibiotic resistance marker gene (hygromycin), which should be avoided, according to current EU regulations (EC 2013a).

5.10.2 Comparative assessment

Increasing grain yield is one of the most valuable agronomic traits and a primary objective in plant breeding. Morinaka et al. (2006) investigated the feasibility of producing high-yielding semi-dwarf rice cultivars by genetic engineering of loss-of-function mutants. Different GM semi-dwarf phenotypes, that were developed and investigated in this research study, underwent a comparative analysis (including morphological traits and yield components) with wild-type plants.

The semi-dwarf phenotypes were generated using a co-suppression strategy: repression of the endogenous *OsBRI1* gene by constitutive expression of a transgenic truncated *OsBRI1* cDNA construct (= lines BKD11, BKD22). The comparative studies resulted in significant differences with respect to the following agronomic endpoints: plant height, culm length, panicle length, grain weight. Malformed leaves were also observed. (For more details on the observed unintended effects please see Chapter 5.10.4.)

Morinaka et al. (2006) did not perform a systematic comparative assessment and the study data especially lack of compositional data. Furthermore, the evaluation of Morinaka et al. (2006) focussed only on traits that are connected with yield, and so, for example, data in relation to changes in the response to different environments (soil, temperature, etc.) were not considered and data in relation to plant pathogens and insect pests were not collected. Therefore, accurate conclusions on the safety, the nutrient profile of high-yielding GM rice cannot be drawn. In particular, no statements are possible assessing the substantial equivalence of the high-yielding GM rice and conventional rice.

In conclusion, the comparative assessment should at least be performed as described by EFSA (2011a). However, as significance and characteristic of unintended effects depend on the different environmental conditions in which plants are grown, it is important that field testing for comparative studies of high-yielding GM plants needs to include a wide range of different environments.

Further field tests and evaluations are necessary in case of a high number of significant differences are observed. In some cases, it may be impossible to find a suitable conventional counterpart due to the morphological changes lead to significant changes in the composition and the phenotype of the plant. This needs to be accounted for in the toxicological and allergological assessment of the GM plant.

5.10.3 Toxicological and allergological assessment

In their study, Morinaka et al. (2006) did not provide any information regarding the toxicological and allergological risk assessment of the high-yielding GM rice varieties developed through co-suppression of a rice *OsBRI1* gene. Furthermore, no other toxicity experiments for GM plants with high yield traits are currently documented in the scientific literature.

Often, high-yielding GM plants are modified by targeting complex growth and physiological processes. This modification usually concerns different classes of plant hormones (e.g. brassinosteroids) for achieving the intended changes by influencing plant development and morphology. But, although simple morphological changes can already be understood at the genetic level, more complex morphological changes are currently not understood, even not in the model plant *Arabidopsis thaliana* for research in plant biology (de Bossoreille de Ribou et al. 2013).

The toxicological and allergological assessment of a high-yielding GM plant, therefore, should account for any changes in the physiology and morphology of the plant. Especially, the potential presence of new substances (e.g. allergens, toxins) and the potential changes in the levels of endogenous constituents (beyond normal variation) should be considered. In any case, data need to be generated to estimate possible risks for human and animal health including long-term effects, and adverse effects on development and reproduction.

5.10.4 Risks associated with pleiotropic effects

Morinaka et al. (2006) studied two research approaches: Firstly, nine loss-of-function mutants were evaluated for their suitability as high yielding cultivars. Secondly, a transgenic rice developed through a co-suppression system for producing brassinosteroid insensitive plants was evaluated for its suitability as high yielding cultivar. Only the second approach was found to be successful.

Pleiotropic effects in knock out mutants
The experiments showed quite distinctive differences between the loss-of-function mutants designed by knock-down of nine *OsBRI1* (*d61*) alleles.

The loss-of-function mutants of the alleles *d61-1* and *d61-2* were found to be weak and produced agronomically useful traits such as semidwarf stature, and elongated neck internodes. In contrast, the severe alleles (*d61-3, d61-4, d61-5, d61-6*) produced severe dwarfisms and also malformations (tortuous leaf blades). In contrast, the remaining three lines (alleles *d61-7, d61-8, d61-9*) were semidwarf phenotypes with erect leaves.

Additionally, the d61-1 and d61-2 plants showed morphological variations concerning the reproductive organs, and also the grain yield was decreased, and the grain number per panicle was reduced.

Most intriguing was the observation that almost all leaves of the d61-1 and d61-2 plants were erect, which is not the case in the wild-type rice. Wild-type plants have leaves that bend away from the vertical axis of the leaf sheath. The d61-7, d61-8, d61-9 lines also had erect leaves, contrary to the d61-3, d61-4, d61-5, d61-6 lines with the malformed leaves.

Because of these results, which varied greatly between the loss-of-function mutants, it was concluded that neither of the nine d61 plant lines seems to be suitable for breeding of high-yielding cultivars (Morinaka et al. 2006).

Pleiotropic effects in transgenic plants constructed by co-suppression
In the same study (Morinaka et al. 2006), the co-suppression of a truncated sense cDNA construct was used to repress endogenous *OsBRI1* expression. This led to brassinosteroid insensitive plants showing erect leaves (lines BKD11, BKD22). Most interestingly, the erect leaf phenotype was seen also in upper leaves that expanded at the reproductive stage, including the flag leaf. Morinaka et al. (2006) concluded that this characteristic should lead to higher yields in the field under high densities.

The following additional effects were discovered during the experimental evaluations of the BKD11 and BKD22 GM plants:

- The degree of bending between leaf blade and leaf sheath was decreased.
- The panicle number per plant were increased.
- The grain weight were slightly increased.

Potential changes in the plant composition were not addressed in this study.

From the authors' point of view, the high variations and observed differences in both experiments suggest that the development of high-yielding rice plant varieties leads to a high number of unintended effects most of which are not understood so far.

Field studies for studying the ecological behaviour of transgenic plants by Zeller et al. (2010) have shown that GM plant risk assessment has to take into consideration the interrelation of environmental factors and possible unintended effects in GM plants, which means that unintended effects of the transgene are highly influenced by environmental factors. It is further demonstrated that, depending on the insertion event, a particular transgene can have large effects on the entire phenotype of a plant and that these effects can sometimes be reversed when plants are moved to another environment (e.g. from the glasshouse to the field). The mechanisms underlying these effects are still unclear.

Especially with agronomic traits like increased yield, this results show the complexity of plant biochemical processes and the factors influencing and determining the physiological status of a plant. The risk assessment should account for these aspects.

5.10.5 Exposure assessment

For the trait "GM plant with improved yield" no exposure assessment was performed because the benefit of the described plant is only to improve agronomic properties. No ingredients are elevated or decreased. Moreover, no protein expression data which could be used for an exposure assessment are available from the study report by Morinaka et al. (2006), and therefore no calculations have been performed.

6 Discussion and conclusions

6.1 Data analysis and categorisation

The second generation of GM plants can be defined as a new group of transgenic plants providing benefits for consumers and industrial applications. Recent progress in genetic engineering has led to the development of a large number of GM plants with different characteristics that belong to this group. Some second generation GM plant events have already reached market maturity, but most of them are still in the phase of development and testing.

Current analysis of USA and EU field trial data led to the classification of second generation GM plants in a categorisation scheme with seven main categories: modification of ingredients, increase of storage time, bioreactors, production of commodities for the industry, modification of processing characteristics, modification of agronomic properties, reduction of anti-nutritive substances. For evaluation of the data and illustration purposes of the different types of second generation GM plant traits, further sub-categories and sub-groups were defined.

The field trial data are the basis for analysing revealed differences in temporal distributions and are indicating trends for GM plants with different specific properties. Distinctive differences between the USA and the EU in relation to second generation GM plant field trialing were observed: the USA data show an increase for the last 10 years (especially in stacked event field testing), while the EU data show a decline; for the last five years, the annual numbers of traits tested in the USA have been ranged from 250 - 360, while in the EU the annual numbers have been about 7 - 12 only.

A potential for authorisation of second generation GM plants exists in the United States for the categories "commodity" (e.g. altered fatty acid content, altered protein content) and "processing" (e.g. altered bread making characteristics, altered grain processing), and for the subgroups "digestibility", "vitamin", "mineral", and "seedlessness".

The trend analysis focusses on the USA field trial data, because several European biotechnology companies have transferred their field testing activities to the United States. Authorisations in the United States, however, may lead to proposals for application of the same GM plant traits in the European Union.

6.2 Risk assessment and applicability of Regulation (EU) No 503/2013 (EC 2013a)

The number of potential applications for second generation GM plants and of the different genetic engineering approaches (introduced genes, targeted metabolic pathways, silencing/suppression methods, etc.) is huge. Hence, the case-by-case principle for the risk assessment of such plants has to be applied taking account of individual characteristics of different types of GM plants and enabling risk assessors to find answers on highly specific questions due to their distinct features.

Basis requirements have been established for GMO risk assessment during the past 25 years: Regulation (EC) No 1829/2003 defines for verification of potential hazards arising from the marketing of GM food and feed, such products need to undergo a risk assessment. The current set of requirements for the risk assessment to be provided in GMO applications is laid down in the Commission Implementing Regulation (EU) No 503/2013. These basis requirements are generally applicable for the risk assessment of second generation GM plants and derived food and feed.

However, the review of the risk assessment of second generation GM plants has revealed a need for amendment of current EU regulation. Aspects addressing special points due to the specific characteristics of second generation GM plants should be considered and more detailed specifications given. Most important aspects are described in the following.

6.2.1 Altered metabolic pathways

In some cases, multiple structural and regulatory genes are used in genetic engineering for modification of plant metabolic pathways leading to the accumulation of nutrients (e.g. sugars) or major changes in nutrient profiles. This may concern any ingredient with a potential industrial benefit or benefit like e.g. sugars, fatty acids, proteins, vitamin, amino acids and minerals. Also, the simultaneous enhancement of ingredients (e.g. vitamins in vegetables or fruits) and the substitution of harmful or unhealthy substances (e.g. gliadins) is a target for newly engineered second generation GM plants.

In relation to targeted and altered metabolic pathways in GM plants, the Regulation (EU) No 503/2013 points out, "*the characteristics of the GM plant trait may trigger further analysis of specific compounds including metabolites of potentially modified metabolic pathways and that the applicant shall consider the inclusion of compounds and justify the selection of these compounds.*"

The authors propose that the inclusion of metabolites of potentially modified metabolic pathways in the comparative assessment should be a requirement and not an option. Many experiments with nutritionally enhanced GM plants have shown that changes in metabolite profiles occurred regularly, and in some cases major changes have been observed (e.g. high β-carotene potatoes showed alterations of pathways of glycolysis, starch synthesis and lipid biosynthesis).

It is, furthermore, recommended that state-of-the-art tools such as omics technologies are developed for the identification and characterisation of such new traits.

6.2.2 Production of new substances

Some of the second generation GM plants may produce new substances that they usually either do not produce at all or only in tiny quantities. Such an example are thaumatin producing GM crops, since thaumatin is a component of the tropical plant *Thaumatococcus daniellii* but no component of the main crops planted worldwide. Up to date, thaumatin expression has been sucessfully applied to e.g. potato, cucumber and tomato.

The safety and nutritional assessment for second generation GM plants producing new substances shall be as strictly as for novel foods. Particularly, a risk-benefit assessment should be carried out.

6.2.3 Concept of substantial equivalence

Regulation (EU) No 503/2013 lays down specifications on how to conduct the comparative analysis helping to identify differences between GM food and feed and their non-GM conventional counterparts with a history of safe use. Usually, an adequate field trial design and statistical method are required for providing a significant database allowing conclusions on the equivalence of a GM plant and derived products as regards compositional and phenotypic characteristics.

From the authors' point of view, GM plants with modified agronomic properties like stress tolerance (drought tolerance, salt tolerance, cold tolerance, etc.) or increased yield traits are not appropriately covered by Regulation (EU) No 503/2013. However, it is important that these GM plants are field tested under specific

conditions (e.g. water-limited environments, different CO_2 concentrations) to accurately measure and evaluate plant performance and other parameters including composition.

Conventional plants cannot be used as reliable comparators under these specific conditions. Therefore, the test design has to be adjusted: GM plants under stress conditions depending on the trait, GM plants under normal conditions, non-GM conventional counterparts under normal conditions. Additional reference varieties have to be included for estimation of the range of natural variation as laid down by Regulation (EU) No 503/2013.

In order to obtain reliable datasets with respect to environment x gene interaction, and to take sufficient account of effects of environmental factors, field trials for GM plants with modified agronomic properties should be conducted at least over two years at the same locations and under comparable conditions.

Another special scenario arises from the development of GM plants that are subject to a substantial modification. In relation to specific requirements on the comparative assessment, and particularly on the selection of comparators, Regulation (EU) No 503/2013 refers to current EFSA guidance (EFSA 2011b). This guidance points out, "The selection of appropriate comparators for the risk assessment of these GM plants with complex modifications may be difficult. When no appropriate comparator is available, the risk assessment should be based primarily on the evaluation of the characteristics of the GM plant and derived products themselves." However, the term "complex modification" is not clearly defined by current EU law or risk assessment guidance, but, in literature, most often associated with "*a specific trait bringing complex changes in the composition of the GM food or feed*" (ADAS 2013).

Regulation (EU) No 503/2013 notes that, where no appropriate conventional counterpart can be identified, a safety and nutritional assessment of the genetically modified food or feed shall be carried out. This assessment should be similar to those for novel foods that do not have conventional counterparts. The key issues for such a risk assessment are laid down in Commission Recommendation (97/618/EC). One key point is that "*the wholesomeness assessment has to take into account not only knowledge of the identity, chemical structure and physico-chemical properties of the Novel Food but also aspects such as source, composition, potential intake based on the proposed use in the general diet, the potential exposure of particularly vulnerable population groups, and the likely effects of processing. The greater the predicted dietary exposure the more extensive the required toxicological testing programme will have to be*" (EC 1997).

It is agreed by the authors that the safety and nutritional assessment for plants with new substances and substantial modifications should be similar to and as strictly as for novel foods. Appropriate animal and *in vitro* studies are to be carried out to assess the toxicological and allergological profile of the GM food/feed.

6.2.4 Toxicology and allergenicity

First generation GM plants either produce substances which have an *a priori* toxic effect (Bt toxins) or allow the application of herbicides at high concentrations. In contrast, second generation GM plants are designed to provide benefits for end consumers and also for industrial applications. Often, enhancement of nutrients is an important feature for these plants aiming at the production of nutritionally valuable foodstuffs.

Regulation (EU) No 503/2013 provides general advice for the risk assessment of nutritionally enhanced GM plants: Depending on the product, information shall be submitted on the composition, level of undesirable substances, nutritional value, the metabolism, and the intended use. Furthermore, the applicant has to consider the submission of additional experimental data. It is important that safety profiles (i.e. profiles established by a substantial battery of quality tests addressing safety aspects (chemistry, chronic toxicity, carcinogenicity, etc.)) for any newly expressed proteins and compounds that are produced by the GM plant at

different levels as compared to its conventional counterpart are established; this to guarantee that potential adverse effects due to the consumption of new substances which do not have a documented history of safe consumption by humans and animals are detected and specified. In relation to the safety of the newly expressed proteins and compounds, Regulation (EU) No 503/2013 mentions the mandatory use of animal studies and other toxicological tests. From the authors' point of view, it is particularly important that studies are conducted for verification that a nutrient or compound as derived from the GM plant bears no risks for causing long-term adverse effects or adverse effects to development and reproduction systems, if this nutrient/compound has no well established safety profile.

In this respect, it is highly important to produce reliable data to demonstrate the absence of unintended alterations of plant metabolic pathways leading to changed levels of low plant metabolites and secondary plant substances, of which there may be little knowledge on the toxicological and allergological safety, or the potential for having anti-nutritional qualities.

Target groups and non-target groups

For GM soybean oil containing high amounts of oleic acid, no target groups can be identified. Based on an exposure assessment considering the intended use of the GM soybean oil, the increased intake of oleic acid and decreased intake of linoleic and palmitic acid does not raise nutritional concerns.

The fatty acid profile of GM soybean oil with high amounts of stearidonic acid is comparable to the fatty acid profile of refined echium oil and *Buglossoides arvensis* oil which are classified as novel foods. No target groups are defined for this GM trait which is a typical example for the genetic engineering of a plant for nutritional purposes. Stearidonic acid is a precursor of the long chain ω-3 eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Both are essential fatty acids and important for human health, since they are known to reduce the risks for coronary heart disease (Mozaffarian et al. 2005). But, it must be secured that there is no overconsumption of ω-3-fatty acids. In addition, the labelling, the correct usage and the recommended dietary intake, has to be assured. It should also be taken into account that there are other ω-3 fatty acid sources like fish, fish oil (used in food supplements) and a lot of novel food products (e.g. oil from microalgae, krill oil) on the market.

The GM plant with a very low content of coeliac-toxic epitopes (gliadin) will be consumed by persons suffering from coeliac disease, so they are the target group. This can lead to changes in consumer behaviour which have to be adequately taken into consideration during the risk assessment, moreover since the transgenic glutenin may represent an allergological risk by itself. GM plants with enhanced vitamin (pro- vitamin) content are "Golden Rice 2" and "Golden Tuber". These plants with increased β-carotene have been developed for countries struggling with vitamin A deficiency, like in Asia and Africa. Based on the performed exposure assessment, no exceedance of the recommended daily intake of retinol-equivalent was reached in the Austrian consumer (non-target) groups. No target group can be attributed to GM plants which express the sweet protein thaumatin. Based on the toxicological information available, no increased risks due to the intake of GM cucumbers producing thaumatin have been identified.

No target groups are identified for GM plants producing thermotolerant enzymes, which are intended for industrial purposes, like production of biofuels and destillation processing. Also, GM plants with high amounts of lysine have no target consumer groups as they are not intended for human consumption. This is also the case for the enhancement of erucic acid in GM rapeseed varieties used for synthesis of nylons and as raw material in the lubricant and oleochemical industry, and products derived from GM plants with increased yield.

In conclusion, it can be argued that different patterns of use and consumption of a food, potential alterations in the total diet of specific population groups have to be taken sufficiently into account during the risk

assessment process of nutritionally enhanced second generation GM plants. This is the case, in particular, when these GM plants are designed for people of certain geographical regions, or people with food sensitivities or food disorders (e.g. allergies).

6.2.5 Exposure assessment

For GM plants with novel traits which provide a nutritional benefit to the consumer, the exposure assessment is a very important part of the risk assessment. For these GM plants with expressed ingredients of interest not only a comparison of the plant carrying the novel trait to a conventional counterpart, but also a comparator of another food product which contains the ingredient of interest should be used for the assessment to introduce the nutritional aspect as well. For example, GM soybean oil MON87769 with high stearidonic acid content is comparable to the fatty acid profile of refined echium oil and *Buglossoides arvensis* oil which are classified as novel foods, due to the circumstance they were not consumed in high amounts before the 15th of May 1997. All of these oils contain high amounts of ω-3-fatty acids which are healthy ingredients regulated under the Health Claim Regulation (EU) No 432/2012 (EFSA 2010e; EFSA 2011d). It must be secured, however, that there is no overconsumption of ω-3-fatty acids.

Although health benefits are associated with high stearidonic acid GM soybean, an oversupply of stearidonic acid from multiple sources may cause adverse effects. To prevent these adverse effects, there should be closer cooperation and better exchange of information between the EFSA Panels on Genetically Modified Organisms (GMO) and Dietetic Products, Nutrition and Allergies (NDA) in relation to the risk assessment of similar food products. Commission Implementing Regulation (EU) No 503/2013 seems to be an appropriate tool for the exposure assessment of novel traits, but the evaluation of the estimated intake of the ingredient(s) of interest is not done in the same way like it is carried out with novel food ingredients.

Therefore, it is recommended for nutritionally enhanced second generation GM plants using the same procedure of intake estimation (food categories) and labelling with respect to health claims like applied to novel foods.

6.2.6 Post-market monitoring

Regulation (EU) No 503/2013 recommends a post-market monitoring plan for nutritionally modified GM plants to confirm the exposure assessment using realistic consumption data for the European population.

Post-market monitoring (PMM) is a risk management tool that should be used on a case-by-case basis to complement the pre-market risk assessment, for example when maximum intake levels are unknown, or when dietary exposure is expected to change due to popularity in the market or perceived health benefits of the trait.

Based on the idea of post market monitoring and to improve the consumer safety, it should be pointed out that in Europe a national nutritional vigilance scheme was established by the French Act on Regional Health (loi Hôpital, Patients, Santé et Territoires) and placed under ANSES's ("French Agency for Food, Environmental and Occupational Health & Safety") responsibility in July 2009. Such a tool is a very important and helpful instrument to monitor such new products and protect consumers.

The goal of this scheme is to improve consumer safety by rapidly identifying potential adverse effects related to consumption of (ANSES 2014):

- food supplements,

- food or drinks fortified with substances for nutritional or physiological purposes (vitamins, minerals, amino acids, or plant extracts) such as energy drinks,
- novel foods and novel food ingredients such as phytosterols, guar gum, and noni juice, and
- products intended for specific population groups (infants, athletes, patients with food intolerances, etc.).

6.2.7 Supply chains - unavoidable commingling / adventitious presence

Second generation GM plants may be used as bioreactors/producers of commodities for non-food/-feed purposes like e.g. detergents, polymers, lubricants, biofuels. Current examples are high erucic acid traits, modified starch traits and glucose isomerase producing plants.

In each case it is necessary to ensure that potentially hazardous crops are prevented from entering supply chains. However, some of the intended products (modified starches or glucose isomerase or other natural enzymes) may not pose high risks in connection with unintended mixing of food supply chains. Other substances like erucic acid are harmful for humans and animals and need higher safety measures.

6.2.8 Risk-benefit assessment

For second generation GM plant traits with modified nutrients a risk benefit assessment should be considered. With respect to these GM plants which provide health benefits, Regulation (EU) 503/2013 notes that altered bioavailability should be taken into account; however, aspects for a risk-benefit assessment are not covered by this regulation (EC 2013a).

The EFSA Scientific Committee has developed a guidance document for health risk-benefit assessment of foods (EFSA 2010b). This guidance focusses on human health risks and human health benefits and does not address social and economic considerations: The Scientific Committee proposes to mirror the risk assessment process by introducing four steps for the benefit assessment: positive health effect identification, positive health effect characterisation, exposure assessment and benefit characterisation. It is further pointed out that this approach will facilitate a transparent comparison of risks and benefits in the risk-benefit assessment (EFSA 2010b).

The importance of the dietary intake measurement (the measure of exposure) as an determinant of the adequacy of the exposure data for risk and benefit assessment is further mentioned by the EFSA Scientific Committee, and it is noted that the positive effect of a nutrient in correcting deficiency may be well established, but only applies to individuals who are deficient (EFSA 2010b).

In this respect, risk-benefit assessments based on standardised methodologies and procedures address not only potential negative effects of a GM food, but can provide scientifically sound data for the characterisation of health benefits in connection with nutrients and new substances produced and elevated by second generation GM plants. An integrated risk-benefit assessment is particularly valuable, since information on both risks and benefits can be helpful to the risk-benefit manager in order to reach a decision on appropriate risk-mitigating measures (Putten et al. 2011).

The authors conclude that risk-benefit assessments can be seen as appropriate instruments for the overall evaluation of advantages and disadvantages in connection with new substances produced by and nutritionally enhanced food derived from second generation GM plants.

7 Recommendations

In the previous chapter, several considerations regarding the risk assessment of second generation GM plants are discussed. Many of these considerations are not appropriately covered by Regulation (EU) No 503/2013. The following recommendations for a comprehensive risk assessment were, therefore, developed from these considerations in a project workshop.

The authors recommend:

- metabolites of pathways that are targeted by the intended modification to be mandatorily included in the comparative assessment.
- additional comparative endpoints in compositional assessments aiming at identification of substances caused by unintended alterations of plant metabolic pathways and produced at elevated levels in plant tissues. Because there may be little knowledge on toxicological and allergological profiles of such substances, comprehensive safety testing will have to be carried out.
- in addition to the conventional counterpart a well established food product containing the ingredient of interest in similar amounts as additional comparator for nutritional safety purposes, if available.
- that GM plants with modified agronomic properties are tested under specific conditions depending on the trait (e.g. water-limited environments, different CO_2 concentrations) to be able to correctly measure and evaluate plant performance and other parameters including composition.
- that field trials for GM plants with modified agronomic properties to be conducted at least over two years at the same locations and under comparable conditions, in order to obtain reliable datasets with respect to environment x gene interaction, and to take sufficient account of effects of environmental factors.
- state of the art tools such as omics technologies to be developed for the identification and characterisation of new traits, especially when metabolic pathways are targeted.
- that adequate safety profiles for any newly expressed proteins and compounds that are produced by the GM plant at different levels as compared to its conventional counterpart are established including methods for the verification of long-term adverse effects or adverse effects to development and reproduction systems.
- particular attention being paid to the toxicological, allergological and nutritional assessment of second generation GM plants designed for specific groups of populations (e.g. people with food disorders), as highly different patterns of use and consumption of a food are to be expected. Target and non-target groups should be identified and potential alterations in the total diet of these population groups taken into account.
- using worst case scenarios for exposure assessments as it is highly unclear how patterns of consumption will change due to the market availability of new GM products.
- that GM plants used as bioreactors and producers of commodities for non-food/feed purposes are prevented from entering supply chains by defining clear confinement and monitoring measures and by establishing safety thresholds. This particularly applies to GM plants producing potentially harmful substances in high quantities.

- post market monitoring (PMM) to be used on a case by case basis as an essential risk management tool.
- that new substances are subject to an annual safety evaluation, because they may be used in food or feed production in high quantities or may be directly consumed. This evaluation should take into consideration the results of Post Market Monitoring (PMM) and any new knowledge generated by research.
- a risk-benefit assessment for nutritionally enhanced second generation GM plants following the rules for novel food, since food derived from second generation GM plants may benefit certain populations, while other populations may be at risk from the same food.

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9 Annex

9.1 GM plant traits field tested in the EU

Table 29: Second generation GM plant traits tested in the EU, sorted by year (trait names)

Trait names	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	Sum
alteration of carbohydrate composition	1	3	3	2	2	5	3	7	5	3	3	3	3	1										**44**
alteration of ethylene biosynthesis							1	4																**5**
alteration of forage quality						1	1																	**2**
alteration of keeping qualities									2															**2**
alteration of lignin biosynthesis				1	2	4	3		1	1	1		2	1			2			1		1	1	**21**
alteration of number of stomata														1										**1**
alteration of oil composition		1			5	6	9	2			1	1	1	1		2		3		1	1			**34**
alteration of photosynthetic properties												1		1		1								**3**
alteration of plant morphology													15											**15**
alteration of ripening characteristics			2	2				2																**6**
alteration of starch biosynthesis				2	9	16	5	12	6	10	2	1	4	3	3	5	1		1					**80**
altered amino acid metabolism								1																**1**
altered distribution of storage metabolites		1							1															**2**

Trait names	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	Sum
altered phosphate metabolism					1	1	1	4																**7**
biofuel																				2				**2**
cold tolerance		1			1	1												1					1	**5**
conversion of phytic acid						1																		**1**
conversion of xylan into xylose					1																			**1**
downregulation of acid invertase								1																**1**
downregulation of amino acid permease								1																**1**
downregulation of amylose synthesis		1	2			4	1	3	1	1	1	1	2	1			2							**20**
downregulation of cinnamoyl CoA reductase				1	2	4	3		1															**11**
downregulation of cinnamyl alcohol dehydrogenase													1											**1**
downregulation of endoglucanase					1																			**1**
downregulation of fructose-1,6-bisphosphatase							1																	**1**
downregulation of glucosinolate									1	1														**2**
downregulation of invertase										1	1													**2**
downregulation of nitrate level			1	2	2	2	1	1	1															**10**
downregulation of nitrite level				1																				**1**
downregulation of o-methyl transferase					1	1			1															**3**

Trait names	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	Sum
downregulation of pectin esterase				2	1																			**3**
downregulation of polyphenol oxydase		1	1		1			1																**4**
downregulation of the photorespiration rate									1															**1**
downregulation of zeaxanthinepoxydase															1									**1**
drought tolerance				1		3	1				2				1	2	1							**11**
dwarf phenotype									1	1														**2**
earlier development of seed embryo																			1					**1**
elevated stomata density															1									**1**
expression of a beta-glucanase																		1						**1**
expression of cyanophycin synthetase																		2	1					**3**
expression of endochitinase																		1						**1**
improved nitrogen assimilation														1								1		**2**
improved photosynthesis															2									**2**
improvement of baking quality					1		1				1													**3**
improvement of digestibility				1																				**1**
improvement of elasticity of flax fibres														1							1	1		**3**
improvement of flowering characteristics					1																			**1**
improvement of malting quality								1			1													**2**

Trait names	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	Sum
improvement of paper production													1											**1**
improvement of photosynthetic performances													1											**1**
improvement of processing quality		1	4	3	5	5	2	3		1														**24**
improvement of starch quality								1	3					4		2								**10**
improvement of storage proteins									1															**1**
improvement of the efficiency of ammonium assimilation/retention												1												**1**
improvement of thermoplastic properties of flax fibres														1							1			**2**
improvement of wood quality													1								1			**2**
Increase of zeaxanthin															1									**1**
increased amino acid content										1			3											**4**
Increased amino acid transporter protein															1									**1**
Increased amino acid uptake into seeds															1									**1**
increased amylopectin content	1	6	4	4	6	8	3	4		2			5	5	4	4	2	2	5		2	1		**68**
increased amylose content														2	3	2	1							**8**
increased beta carotene content													1											**1**

Trait names	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	Sum
increased carotenoid content												1												**1**
increased cell wall thickness						1																		**1**
increased erucic acid content						1	2																	**3**
increased fitness						1																		**1**
increased food quality												1												**1**
increased glutaninic complex content														1										**1**
increased laurate content		1		1	6	7	8	1		1														**25**
increased metabolism of reducing sugars in tubers															1									**1**
increased nutritional value		2	1	1		2	1																	**7**
increased oil content														1	2	2	2				2			**9**
increased protein content																1			1					**2**
increased starch content															1			1	1	2	1	2		**8**
increased stearate content		1		1	5	2	1	1																**11**
increased storage		1	3	2	1			1																**8**
increased tuber yield														1										**1**
increased vitamin content																						1	2	**3**
increased yield		1			1	3		2	1				15		1	1				2	3	2	1	**33**
induction of parthenocarpic fruit												2												**2**
inhibition of flowering											1													**1**
inhibition of NAD-malic enzyme								1	1															**2**
inhibition phosphoglucomutase										1														**1**
inhibition threonine synthase										1														**1**

Trait names	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	Sum
low content of coeliac-toxic epitopes																							1	**1**
modification of plant architecture, flowering and fruiting behaviour																1		1						**2**
multiple testing of traits involved in regulation of autumn phenology																						2		**2**
overexpression of photosynthetic proteins											1							1						**2**
reduction of antinutritional effect of phytic acid							2																	**2**
reduction of blackspot		1	1	1	1			1																**5**
reduction of phosphate pollution							1																	**1**
reduction of shattering of mature pods						1	1	2	2	2	1													**9**
reduction of stearic acid content									1															**1**
secretion of alpha-amylase						1			1															**2**
silencing of SDD1 gene															1									**1**
stimulation of growth rate								1		1								1	2					**5**
stress tolerance						1	3			2			3											**9**
suppression of shade avoidance				1	1	1				1														**4**
synthesis of a spider silk-elastin fusion protein												1		1										**2**
synthesis of ACC synthase				1	1			3																**5**
synthesis of ADP glucose pyrophosphorylase						1	2	1																**4**
synthesis of albumin					1																			**1**

Trait names	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	Sum
synthesis of arginine decarboxylase									1															**1**
synthesis of asparagine					1	1	3	1																**6**
synthesis of branching enzymes					1		1	2		1														**5**
synthesis of fructan				2	1	1	1	2	3		1					1								**12**
synthesis of fructosyltransferase			1	2	1	2	2						1											**9**
synthesis of gibberellin												1												**1**
synthesis of glucose isomerase						1																		**1**
synthesis of glutamine synthetase								1				1												**2**
synthesis of glycogen branching enzyme							1																	**1**
synthesis of hexokinase II							1																	**1**
synthesis of high molecular weight glutenin											1													**1**
synthesis of human albumin									1									1						**2**
synthesis of inorganic pyrophosphatase							1																	**1**
synthesis of invertase						1	4																	**5**
synthesis of kestose								1																**1**
synthesis of leghemoglobin													1											**1**
synthesis of levan sucrase				1	1	4	2																	**8**
synthesis of lysine									1															**1**
synthesis of maltose binding protein							1																	**1**
synthesis of metallothionein																	1							**1**

Trait names	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	Sum
synthesis of methionine/lysine rich protein		2	1																					**3**
synthesis of nitrate reductase			1	2	3	3	3	1	1	2														**16**
synthesis of nitrite reductase				1	1	1	2																	**5**
synthesis of non-plant carbohydrates												1												**1**
synthesis of nystose								1																**1**
synthesis of oligogalacturonate lyase				1																				**1**
synthesis of pectate lyase						1		2																**3**
synthesis of phytase							2				1										1	1		**5**
synthesis of phytochrome A				1	1	1			1															**4**
synthesis of phytochrome B							1		1															**2**
synthesis of polygalacturonase		1	1	2	3	2		3																**12**
synthesis of putrescine methyl transferase							1																	**1**
synthesis of pyrophosphate						1																		**1**
synthesis of sucrose fructosyltransferase										1														**1**
synthesis of sucrose phosphate						2		1																**3**
synthesis of sucrose-isomerase												1												**1**
synthesis of superoxide dismutase							1			1														**2**

Trait names	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	Sum
synthesis of thaumatin																		2					1	**3**
synthesis of the bicolor coding sequence															1									**1**
synthesis of threonine					1																			**1**
synthesis of trehalose						1		1																**2**
synthesis of trehalose-6-phosphate				1																				**1**
synthesis of tryptophan-2-monoxygenase							2		5	2														**9**
use as bioreactors					1																			**1**
Sum	**2**	**25**	**26**	**43**	**74**	**106**	**86**	**78**	**46**	**38**	**19**	**17**	**60**	**27**	**25**	**24**	**12**	**17**	**12**	**8**	**13**	**12**	**7**	**777**

9.2 GM plant traits field tested in the United States

Table 30: Second generation GM plant traits tested in the USA, sorted by year (trait names)

Trait names	1988	1989	1990	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	Sum
Accelerated Ripening																			1	1							**2**
Acid Invertase Activity Reduced																										1	**1**
Alkaloid Content Decreased															1		2			2	1	1			1		**8**
Alkaloids Increased																				2							**2**
Alteration Of Metabolic Pathways (Amino Acids)																						1					**1**
Altered Alkaloid																									1		**1**
Altered Amino Acid									3		2		1	12	12	16	16	35	24	26	16	15	12	11	10	14	**225**
Altered Auxin Metabolism						1																					**1**
Altered Biomass Processing																						3				2	**5**
Altered Bread Making Characteristics																1			1	1	1				1	2	**7**
Altered Carbohydrate Metabolism						11	29	38	15	26	56	27	8	16	13	4		5	5	1	5	3	4	6	9	13	**294**
Altered Carotenoid Content							1		3	4	3	6							2		1			2	3	2	**27**
Altered Carotenoid Metabolism								1						1								1		1			**4**
Altered Color													2	1	3	4		2	1	1							**14**
Altered Composition																				1							**1**
Altered Digestibility														4	2	1		3	4	2	8	19	16	14	5	4	**82**
Altered Erucic Acid															1												**1**
Altered Ethylene Metabolism										1				1		1		2					2	3			**10**
Altered Fatty Acid																			1			3	7	7	2		**20**
Altered Fatty Acid Content						1	3	2	3	1	2	2	2	3	1		8	10	8	3	15	19	19	14	14	20	**150**
Altered Feed Quality													4	8	5	2	7	9	16	9	22	31	26	22	28	30	**219**
Altered Fiber Quality								1										2	6		9	8	9	5	8	4	**52**
Altered Fiber Strength							1	5	7	1	1																**15**
Altered Flavonoid Content																		2	2	2							**6**
Altered Flavor									1										2	3	4	3	4	4	2	1	**24**
Altered Food Quality																		1	4	1	3	7	3	2	4	1	**26**
Altered Fruit Color																			1	1		2	1	1	1	1	**8**

Trait names	1988	1989	1990	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	Sum
Altered Fruit Quality										1			1			1	1	2	2								**8**
Altered Fruit Ripening	1	2	4	6	12	25	41	31	16	21	22	15	10	4	2	1			2	3		4	1	1	1		**225**
Altered Fruit Sugar							1	1	2	2	1	1	1		1	1	1	2	1								**15**
Altered Grain Hardness																				1	1						**2**
Altered Grain Processing													4	2			2	3	2	1	2	3	1			3	**23**
Altered Growth Rate																	1										**1**
Altered HMW Glutenin Seeds Storage Protein Content																			1								**1**
Altered Kernel Development																		1	1	1	1		4				**8**
Altered Lignin Biosynthesis											1			1	1		8	13	5	4	2						**35**
Altered Lipid Profile																	1	5	4		7	6	6	3			**32**
Altered Lysine Content															10	1	2	33	2	1	2						**51**
Altered Metabolism			1																1								**2**
Altered Morphology																	4				1						**5**
Altered Nitrogen Metabolism								1	3	3	6	7	4	4	1	1	1	1	2	1		3	8				**46**
Altered Nutritional Quality							3	2	2	1	2		1			3	1	8	3		2	6	6	3	7	6	**56**
Altered Oil				2	4	10	17	24	10	18	17	15	8	18	18	28	29	45	62	63	59	36	32	18	17	8	**558**
Altered Oil Content																				8	3	7	16	25	19	18	**96**
Altered Oleic Acid Content															1		1	3	1	1	1	4	3	2		1	**18**
Altered Oxidative Stress Response																			2								**2**
Altered Pigment Composition															8												**8**
Altered Pigment Metabolism						1		2	11	1	2																**17**
Altered Plant Development																6		2		1							**9**
Altered Polyamine Metabolism								1	1	2			1	1			1	1									**8**
Altered Polyphenol Oxidase Content															2	1	1	1	1								**6**
Altered Processing Characteristics								5	1											2	6	5	5	8	12	34	**78**
Altered Protein							51	45	9	21	31	6	5	9	11	8	17	23	8	1	5	6	10	6	9	7	**288**
Altered Protein Content																			3	3			4	9	12	12	**43**

Trait names	1988	1989	1990	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	Sum
Altered Seed Composition				1		1	1	2	6	2	8	9	4	9	25	31	46	46	21	21	27	25	18	17	19	25	**364**
Altered Senescence												1	1		2	2			2	1							**9**
Altered Sorbitol Content																			1	1	1			1			**4**
Altered Starch Content																		2	2	2	3	14	7	9	12	11	**62**
Altered Starch Metabolism					1				1			1	1	3	14	7	7	2	7	7	8	11	9	7	9	7	**102**
Altered Sterols												1						1	1								**3**
Altered Storage Protein				2	6	16		4	1	1	1	4	8	16	5	5	3	4	1				3	3	3		**86**
Altered Sugar Content														1				2	1				2				**6**
Altered Sugar Metabolism																				2	1						**3**
Altered Sugar Storage																							4	12	3	1	**20**
Altered Tocopherol Content																				1							**1**
Altered Wood Chemicals																					1			1			**2**
Altered Wood Quality																			6								**6**
Amount Of Sugars In The Plant Cell Wall Increased																									1		**1**
Amylose Increased																						1	3				**4**
Anthocyanin Increased										1	1													4	1		**7**
Antioxidant Enzyme Increased											1	1	1										1	2			**6**
Antiprotease Producing														1													**1**
B-1,4-Endoglucanase							1																				**1**
Barren Stalk 1-Independent Meristem Formation																				1							**1**
Biologically Safe Wheat Lines For Patients With Coeliac Disease																								1	1	1	**3**
Bruising Reduced										3	24	11	11	1	1	1	2										**54**
Cadmium Partitioning																	1	1	4	2	1						**9**
Caffeine Content Decreased												1															**1**
Carbohydrate Content Increased				1																		1			1	2	**5**
Carotene Content Increased															1				1		3	1	1	2	2		**11**
Carotenoid Content Increased																						2	2				**4**
Catalase Content Decreased														1													**1**
Cell Wall Altered												3						2	4		3	6	3	1			**22**

Trait names	1988	1989	1990	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	Sum
Cold Intolerant																			1								**1**
Cold Tolerance				1	1	2			1									2	3	2							**12**
Cyanogen Content Decreased																						1			1		**2**
Cyanogen Reduction																							1				**1**
Cyanogenesis Increase																				1							**1**
Disulfides Decreased											1																**1**
Drought Tolerance																		3	5	1	2						**11**
Dry Matter Content Increased											1	1											1	2	2	3	**10**
Dwarfed															8			1									**9**
Ethylene Production Reduced							2			2	2	3	1			2		1	2	1	1		1	2	1		**21**
Fatty Acid Content Increased																					1			1	1	1	**4**
Flavonoid Content Decreased																		2					1			1	**4**
Flower And Fruit Abscission Reduced							1	1																			**2**
Fructan Accumulation																					1	1	1				**3**
Fruit Firmness Decreased																					2	2					**4**
Fruit Firmness Increased							2	1									2			1	2	2	1	1	1	1	**14**
Fruit Invertase Content Decreased							1																				**1**
Fruit Pectin Esterase Content Decreased							2													1					1	1	**5**
Fruit Polygalacturonase Content Decreased							3	1													2	2	1	1	1	1	**12**
Fruit Polygalacturonase Content Increased																					2	2			1	1	**6**
Fruit Softening Enhanced																			4						1	1	**6**
Fruit Softening Reduced											1	3						1	2	2							**9**
Fruit Solids Increased							7	4	4	2		1						1									**19**
Fruit Sweetness Increased										1																	**1**
Fumonisin Degradation													4		2		3	2									**11**
Fusarium																				1							**1**
Germination Increased																	3										**3**

Trait names	1988	1989	1990	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	Sum
Glutenin Increased																1		1		1	1	2	1		2		**9**
Heat Stable Glucanase Produced											1	4	2	2	3	1	1	1	1	1							**17**
Heat Tolerant															1				1	2							**4**
High Folate																									1	1	**2**
Higher Polyamine Content																			1								**1**
Hydroxynitrile Lyase (Hnl) Content Increased																				1							**1**
Increase Free Amino Acids																							1				**1**
Increased Germination Rate																				1							**1**
Increased Grain Quality																				2							**2**
Increased Phosphorus											2	3															**5**
Increased Triacylglycerol Content																									1	1	**2**
Increased Tuber Set																									1		**1**
Industrial Enzymes Produced										1																	**1**
Iron Absorption Enhanced																				1	1	3	1				**6**
Iron Bioavailability Enhanced																							1	1			**2**
Iron Content Increased															1						1		2	1		1	**6**
Larger Fruit													1														**1**
Leaf Senescence Delayed													3	1	1												**5**
Lignin Content Decreased											1		1	2	1	9	4		1		4	19	17	11	14	23	**107**
Linolenic Acid Produced																		1	3								**4**
Lysine Content Increased		1					3	2	6	6	11	15	5	4	28	17	26	12	26	3	5	10	7	4	4	4	**199**
Melanin Produced In Cotton Fibers										1	1																**2**
Methionine Content Increased					2	6	11	5	3	2	6	4	2	2				3								1	**47**
Methylbenzoate Decreased																		1									**1**
MiR156 Activity Decreased																						1					**1**
Nicotine Content Decreased												1		14	7	1	5	9	10		1						**48**
Nitrogen Use																							1				**1**
No Known Phenotype																				1							**1**
Non-Browning																				4				1			**5**
Normal Pollen Transmission																									1	1	**2**

Trait names	1988	1989	1990	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	Sum
Normal To Severely Chlorotic And Stunted																										1	**1**
Nornicotine Accumulation Reduced																				1		1					**2**
Novel Protein Produced												1	2	1			1										**5**
Oil Content Increased																1		1	12	6	17	22	18	15	13	16	**121**
ω-3 Fatty Acids Produced																		1	2	1				1	2	3	**10**
Pectin Esterase Content Decreased							1		2	1	1																**5**
Phospholipase D Suppressed																			1								**1**
Phosphorous Uptake Enhanced																					2						**2**
Photosynthesis Enhanced											2	1					1	5									**9**
Phytate Decreased												1	5	2	2		1	2	3	2	4	4	5	1			**32**
Phytoene Synthase Activity Increased							1																				**1**
Polygalacturonase Content Decreased							2	5						1						1							**9**
Polyphenol Oxidase Content Decreased																				4	2		1	2	1	2	**12**
Polyphenol Oxidase Content Increased																										1	**1**
Program Cell Death Inhibited																	1										**1**
Protein Content Increased												1		1		1	3	3	6	2	4	5	5	4	3	2	**40**
Proteinase Inhibitors Content Constitutive							1																				**1**
Random Insertions, No Known Phenotype																				1	1						**2**
Reduction Of Cyanogens																							1				**1**
Rubber Yield Increased													1								2						**3**
Salt Tolerance																		1	2	1							**4**
Saturated Fatty Acids Decreased																			1								**1**
SDA ω-3 Fatty Acid																			3								**3**
Secondary Metabolite														2													**2**

Trait names	1988	1989	1990	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	Sum
Increased																											
Seed Number Increased				1																							**1**
Seed Set Reduced										1								1									**2**
Seed Size Increased												2		2	2		2	1	2	1	3				1	1	**17**
Seed-Gossypol Decreased																						2	1	1	1	2	**7**
Seedlessness Increased																								1	1		**2**
Shikimate Dehydrogenase Overexpression																										1	**1**
Softer Endosperm																			2	11	4	2	2	1		1	**23**
Solids Increased					2	11	5	9	13	4	3	10	6		1	1	1	1	1	1	1	1	2	1			**74**
Stanol Increased													6														**6**
Starch Content Decreased							1				1				1									2			**5**
Starch Content Increased												2	5	13	6	6	2	7	3		3	4	3	1			**55**
Stearic Acid Content Increased																						1					**1**
Stearidonic Acid																							5				**5**
Steroidal Glycoalkaloids Decreased										2	2	1	3	3	2	1	1										**15**
Sterols Increased					1							1															**2**
Stress Tolerance																				1							**1**
Sucrose Synthase Antisense For Seedlessness																								1			**1**
Sugar Alcohol Content Increased												2	2		1												**5**
Sugar Content Increased																						1			1	1	**3**
Thermostable Protein Produced									1	1																	**2**
Tryptophan Content Increased								1		1	2	5	4	9		14	7	12	8	2							**65**
Tuber Solids Increased					2	4	6	5	1																		**18**
Tyrosine Content Increased				1																							**1**
Visual Marker														1			1										**2**
Vitamin A Content Increased																						2		1	1		**4**
Vitamin B19 (Folic Acid) Content Increased																						1	1				**2**
Vitamin C Content																1			1		1	1	1		1		**6**

Trait names	1988	1989	1990	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	Sum
Increased																											
Vitamin E Content Increased																				2	1	5	1				**9**
Wax Ester Production In Seed																							1				**1**
Wheat Gluten Protein Expressed In Kernel																									1	4	**5**
Yield Increased											2	4	1	10	19	39	12	5	1	1		5	8				**107**
Zinc Content Increased																					2						**2**
	1	**3**	**5**	**15**	**31**	**89**	**198**	**199**	**126**	**135**	**221**	**177**	**132**	**187**	**227**	**221**	**240**	**355**	**340**	**250**	**298**	**359**	**345**	**284**	**278**	**308**	**5024**

9.3 GM plant traits (EU, USA) field tested only 2004/2009 and later

The following tables (Table 31 and Table 32) list the trait names that appeared only in the year 2004/2009 and later indicating currentness of specific traits. The attribute "first appearance in the year 2004/2009 or later" is indicated by a green "YES" in the tables. The information provided in these tables is further discussed in Chapter 4.5.

Table 31: EU field trials - GM plant traits with first appearance after 2004 or 2009

Trait names	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	Sum	2009 and later	2004 and later
biofuel							2				2	YES	YES
earlier development of seed embryo						1					1	YES	YES
increased vitamin content									1	2	3	YES	YES
low content of coeliac-toxic epitopes										1	1	YES	YES
alteration of number of stomata	1										1	NO	YES
downregulation of zeaxanthinepoxydase		1									1	NO	YES
elevated stomata density		1									1	NO	YES
expression of a β-glucanase					1						1	NO	YES
expression of cyanophycin synthetase					2	1					3	NO	YES
expression of endochitinase					1						1	NO	YES
improved nitrogen assimilation	1								1		2	NO	YES
improved photosynthesis		2									2	NO	YES
improvement of elasticity of flax fibres	1							1	1		3	NO	YES
improvement of thermoplastic properties of flax fibres	1							1			2	NO	YES
Increase of zeaxanthin		1									1	NO	YES
Increased amino acid transporter protein		1									1	NO	YES
Increased amino acid uptake into seeds		1									1	NO	YES
increased amylose content	2	3	2	1							8	NO	YES
increased glutaninic complex content	1										1	NO	YES
increased metabolism of reducing sugars in tubers		1									1	NO	YES
increased oil content	1	2	2	2				2			9	NO	YES
increased protein content			1			1					2	NO	YES
increased starch content		1			1	1	2	1	2		8	NO	YES
increased tuber yield	1										1	NO	YES
modification of plant architecture, flowering …			1		1						2	NO	YES

Trait names	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	Sum	2009 and later	2004 and later
silencing of SDD1 gene		1									1	NO	YES
synthesis of metallothionein				1							1	NO	YES
synthesis of thaumatin					2					1	3	NO	YES
synthesis of the bicolor coding sequence		1									1	NO	YES

Table 32: USA field trials - GM plant traits with no appearance before 2004 or 2009

Trait names	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	Sum	2009 and later	2004 and later
Acid Invertase Activity Reduced										1	1	YES	YES
Alteration Of Metabolic Pathways (Amino Acids)						1					1	YES	YES
Altered Alkaloid									1		1	YES	YES
Altered Biomass Processing						3				2	5	YES	YES
Altered Sugar Storage							4	12	3	1	20	YES	YES
Amount Of Sugars In The Plant Cell Wall Increased									1		1	YES	YES
Amylose Increased						1	3				4	YES	YES
Carotenoid Content Increased						2	2				4	YES	YES
Cyanogen Content Decreased						1			1		2	YES	YES
Cyanogen Reduction							1				1	YES	YES
High Folate									1	1	2	YES	YES
Increase Free Amino Acids							1				1	YES	YES
Increased Triacylglycerol Content									1	1	2	YES	YES
Increased Tuber Set									1		1	YES	YES
Iron Bioavailability Enhanced							1	1			2	YES	YES
MiR156 Activity Decreased						1					1	YES	YES
Nitrogen Use							1				1	YES	YES
Normal Pollen Transmission									1	1	2	YES	YES
Normal To Severely Chlorotic And Stunted										1	1	YES	YES
Polyphenol Oxidase Content Increased										1	1	YES	YES
Reduction Of Cyanogens							1				1	YES	YES
Seed-Gossypol Decreased						2	1	1	1	2	7	YES	YES
Seedlessness Increased								1	1		2	YES	YES
Shikimate Dehydrogenase Overexpression										1	1	YES	YES
Stearic Acid Content Increased						1					1	YES	YES
Stearidonic Acid							5				5	YES	YES
Sucrose Synthase Antisense For Seedlessness								1			1	YES	YES
Sugar Content Increased						1			1	1	3	YES	YES
Vitamin A Content Increased						2		1	1		4	YES	YES

Trait names	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	Sum	2009 and later	2004 and later
Vitamin B19 (Folic Acid) Content Increased						1	1				2	YES	YES
Wax Ester Production In Seed							1				1	YES	YES
Wheat Gluten Protein Expressed In Kernel									1	4	5	YES	YES
Accelerated Ripening			1	1							2	NO	YES
Alkaloids Increased				2							2	NO	YES
Altered Composition				1							1	NO	YES
Altered Fatty Acid			1			3	7	7	2		20	NO	YES
Altered Flavonoid Content		2	2	2							6	NO	YES
Altered Food Quality		1	4	1	3	7	3	2	4	1	26	NO	YES
Altered Fruit Color			1	1		2	1	1	1	1	8	NO	YES
Altered Grain Hardness				1	1						2	NO	YES
Altered Growth Rate	1										1	NO	YES
Altered HMW Glutenin Seeds Storage Protein Content			1								1	NO	YES
Altered Kernel Development		1	1	1	1		4				8	NO	YES
Altered Lipid Profile	1	5	4		7	6	6	3			32	NO	YES
Altered Morphology	4				1						5	NO	YES
Altered Oil Content				8	3	7	16	25	19	18	96	NO	YES
Altered Oxidative Stress Response			2								2	NO	YES
Altered Protein Content			3	3			4	9	12	12	43	NO	YES
Altered Sorbitol Content			1	1	1			1			4	NO	YES
Altered Starch Content		2	2	2	3	14	7	9	12	11	62	NO	YES
Altered Sugar Metabolism				2	1						3	NO	YES
Altered Tocopherol Content				1							1	NO	YES
Altered Wood Chemicals					1			1			2	NO	YES
Altered Wood Quality			6								6	NO	YES
Barren Stalk 1-Independent Meristem Formation				1							1	NO	YES
Cadmium Partitioning	1	1	4	2	1						9	NO	YES
Cold Intolerant			1								1	NO	YES
Cyanogenesis Increase				1							1	NO	YES
Drought Tolerance		3	5	1	2						11	NO	YES

Trait names	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	Sum	2009 and later	2004 and later
Fatty Acid Content Increased					1			1	1	1	4	NO	YES
Flavonoid Content Decreased		2					1			1	4	NO	YES
Fructan Accumulation					1	1	1				3	NO	YES
Fruit Firmness Decreased					2	2					4	NO	YES
Fruit Firmness Increased				1	2	2	1	1	1	1	9	NO	YES
Fruit Polygalacturonase Content Increased					2	2			1	1	6	NO	YES
Fruit Softening Enhanced			4						1	1	6	NO	YES
Fusarium				1							1	NO	YES
Germination Increased	3										3	NO	YES
Higher Polyamine Content			1								1	NO	YES
Hydroxynitrile Lyase (Hnl) Content Increased				1							1	NO	YES
Increased Germination Rate				1							1	NO	YES
Increased Grain Quality				2							2	NO	YES
Iron Absorption Enhanced				1	1	3	1				6	NO	YES
Linolenic Acid Produced		1	3								4	NO	YES
Methylbenzoate Decreased		1									1	NO	YES
No Known Phenotype				1							1	NO	YES
Non-Browning				4				1			5	NO	YES
Nornicotine Accumulation Reduced				1		1					2	NO	YES
ω-3 Fatty Acids Produced		1	2	1				1	2	3	10	NO	YES
Phospholipase D Suppressed			1								1	NO	YES
Phosphorous Uptake Enhanced					2						2	NO	YES
Polyphenol Oxidase Content Decreased				4	2		1	2	1	2	12	NO	YES
Program Cell Death Inhibited	1										1	NO	YES
Random Insertions, No Known Phenotype				1	1						2	NO	YES
Salt Tolerance		1	2	1							4	NO	YES
Saturated Fatty Acids Decreased			1								1	NO	YES
SDA ω-3 Fatty Acid			3								3	NO	YES
Softer Endosperm			2	11	4	2	2	1		1	23	NO	YES
Stress Tolerance				1							1	NO	YES

Trait names	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	Sum	2009 and later	2004 and later
Vitamin E Content Increased				2	1	5	1				9	NO	YES
Zinc Content Increased					2						2	NO	YES

9.4 Literature research (results)

9.4.1 Scopus

Table 33: Literature research - Scopus

search term	2009 - 2013 reviews	2009 - 2013 articles and reviews	all years reviews	all years articles and reviews
abiotic stress	69	732	150	1131
allergen downregulation	0	0	0	0
altered amino acid	0	1	0	1
altered life	0	0	0	0
altered metabolism	2	6	2	7
altered storage time	0	0	0	0
amino acid/s	42	1608	157	4436
amino acid metabolism	4	17	10	42
biofuel/s	31	130	41	156
biopharmaceutical/s	25	55	42	105
carbohydrate/s metabolism	3	94	21	280
carbohydrate/s	15	243	56	703
downregulation	0	67	5	149
enhanced food/s	3	3	3	5
enriched food/s	0	0	0	0
fatty acid/s	22	275	86	699
fatty acid metabolism	1	7	11	35
food allergen downregulation	0	0	0	0
functional food	3	13	13	33
increased lysine	0	3	0	7
increased oil content/s	1	1	1	2
increased protein/s	0	8	0	16
increased starch	0	3	1	8
mineral/s	16	63	34	157
molecular pharming	2	10	2	10
novel food	0	4	0	0
novel traits	3	13	10	32
nutrient enhancement	0	0	0	0
nutritionally enhanced	0	0	0	0
second generation	0	0	0	0
stress resistance	5	64	14	111
stress tolerance	43	495	99	863
third generation	0	0	0	0
vitamin/s	15	104	48	254
allergen/s	10	71	42	163
protein/s	357	6466	1043	16421
lysine	6	88	21	219
threonine	5	150	18	321
starch	9	202	605	33

oil content/s	6	36	6	55
nutrient/s	39	219	86	506
nutritionally	6	19	12	53
pharmaceutical/s	86	199	178	422
cold	19	419	50	861
nitrogen	14	244	45	607
increased yield	5	16	9	28
flavor/s	5	22	12	63
lignin	13	189	35	428
carotenoid/s	14	118	29	282
glutenin	0	26	4	85
ethylene	12	296	51	774
sum	**911**	**12799**	**3052**	**30563**

9.4.2 PubMed

Table 34: Literature research - PubMed

search term	2009 - 2013 reviews	2009 - 2013 articles + reviews	all years reviews	all years articles and reviews
abiotic stress	28	322	46	475
allergen downregulation	0	0	0	0
allergen/s	10	63	32	137
altered amino acid	0	0	0	0
altered life	0	0	0	1
altered metabolism	1	4	1	5
altered storage time	0	0	0	0
amino acid metabolism	8	1	0	18
amino acid/s	13	542	53	1546
biofuel/s	22	73	31	91
biopharmaceutical/s	18	34	33	73
carbohydrate/s	6	85	16	259
carbohydrate/s metabolism	2	16	5	62
carotinoid/s	0	0	0	0
cold	13	282	22	547
downregulation	0	56	2	129
enhanced food/s	2	3	4	6
enriched food/s	0	0	0	1
ethylene	5	245	22	607
fatty acid metabolism	0	3	2	12
fatty acid/s	13	201	57	505
flavor/s	10	1	12	42
food allergen downregulation	0	0	0	0
functional food	0	3	3	9
glutenin	5	14	1	45

increased lysine	0	3	0	6
increased oil content/s	0	1	2	0
increased protein/s	0	8	0	16
increased starch	1	8	1	8
increased yield	2	7	4	13
lignin	5	124	17	266
lysine	3	67	14	161
mineral/s	8	41	24	111
molecular pharming	2	8	2	10
nitrogen	9	164	28	362
novel food	0	3	3	10
novel traits	0	8	2	14
nutrient enhancement	0	0	0	0
nutrient/s	17	145	64	357
nutritionally	6	22	19	70
nutritionally enhanced	0	1	0	1
oil content/s	4	29	7	47
pharmaceutical/s	41	110	106	256
protein/s	185	3806	539	9594
second generation	3	16	8	54
starch	6	172	21	460
stress resistance	2	37	5	54
stress tolerance	28	318	46	516
third generation	0	5	2	12
threonine	0	49	7	138
vitamin/s	9	62	43	174
sum	**487**	**7162**	**1306**	**17280**

9.4.3 United States patents

Table 35: Literature research - United States patents

search term	1976 to present
abiotic stress	7
allergen downregulation	0
allergen	0
altered amino acid	0
altered life	0
altered metabolism	0
altered storage time	0
amino acid/s	7
amino acid metabolism	0
biofuel	1
biopharmaceutical/s	4
pharmaceutical/s	24
carbohydrate/s	12

carbohydrate metabolism	2
downregulation	0
enhanced food/s	0
enriched food/s	0
fatty acid/s	84
fatty acid metabolism	0
functional food	0
lysine	9
threonine	8
oil content	11
omega	8
protein/s	753
protein/s [GM plant/s]	35
increased protein	0
sugar/s	37
starch	54
increased starch	0
mineral/s	0
iron	5
glutenin	2
color	5
flavor	5
ripening	17
molecular pharming	0
novel food	0
novel trait/s	0
nutrient	3
nutrient enhancement	0
nutritionally	3
increased yield	11
lignin	11
nutritionally enhanced	0
second generation	0
stress resistance	7
stress tolerance	19
ethylene	13
cold	16
nitrogen	13
third generation	0
carotene	0
carotenoid	2
vitamin/s	1
sum	**1189**

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This report presents an in-depth discussion of risk assessment issues regarding genetically modified (GM) plants of the „second generation“. Such GM plants, in contrast to herbicide tolerant and insect resistant (first generation) GM plants, can provide direct benefits for consumers by generating high levels of certain nutrients or by reducing anti-nutrients. They can also facilitate industrial applications by producing commodities, chemicals or specific enzymes. The sources for this study include data from authorisation, field trial and literature databases of GM plants for the last 25 years. The report concludes with recommendations for amendment of existing standards for the risk assessment and food safety evaluation of „second generation“ GM plants.