

SCIENTIFIC OPINION

Scientific opinion addressing the safety assessment of plants developed through cisgenesis and intragenesis¹

EFSA Panel on Genetically Modified Organisms (GMO)^{2,3}

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ABSTRACT

The European Commission requested that the EFSA Panel on Genetically Modified Organisms deliver a scientific opinion related to risk assessment of cisgenic and intragenic plants. The EFSA GMO Panel considers that the *Guidance for risk assessment of food and feed from genetically modified plants* and the *Guidance on the environmental risk assessment of genetically modified plants* are applicable for the evaluation of food and feed products derived from cisgenic and intragenic plants and for performing an environmental risk assessment and do not need to be developed further. It can be envisaged that on a case-by-case basis lesser amounts of event-specific data are needed for the risk assessment. The EFSA GMO Panel compared the hazards associated with plants produced by cisgenesis and intragenesis with those obtained either by conventional plant breeding techniques or by transgenesis. The Panel concludes that similar hazards can be associated with cisgenic and conventionally bred plants, while novel hazards can be associated with intragenic and transgenic plants. The Panel is of the opinion that all of these breeding methods can produce variable frequencies and severities of unintended effects. The frequency of unintended changes may differ between breeding techniques and their occurrence cannot be predicted and needs to be assessed case by case. Independent of the breeding method, undesirable phenotypes are generally removed during selection and testing programmes by breeders. The risks to human and animal health and the environment will depend on exposure factors such as the extent to which the plant is cultivated and consumed.

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KEY WORDS

Cisgenic, cisgenesis, intragenic, intragenesis, transgenic, GM plant

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SUMMARY

Following a request from the European Commission (EC), the EFSA Panel on Genetically Modified Organisms (GMO) was requested to deliver a scientific opinion on plants developed through cisgenesis and intragenesis in terms of the risks they might pose and the applicability of the existing guidance documents for their risk assessment. The mandate included two specific questions:

1. Determine whether there is a need for new guidance or whether the existing guidance on risk assessment should to be updated or further elaborated, in anticipation of the placing of products on the market through the application of the listed techniques.
2. What are the risks in terms of impact on humans, animals and the environment that the eight techniques listed could pose, irrespective of whether or not they fall under the GMO legislation? This latter request should consider the most recent scientific literature and knowledge of plant breeding experts and compare plants obtained by these new techniques with plants obtained by conventional plant breeding techniques and secondly with plants obtained with currently used genetic modification techniques.

The EFSA GMO Panel considers that the Guidance for risk assessment of food and feed from genetically modified plants (EFSA, 2011) and the Guidance on the environmental risk assessment of genetically modified plants (EFSA, 2010) are applicable for the evaluation of food and feed products derived from cisgenic and intragenic plants and for performing an environmental risk assessment and do not need to be developed further. It can be envisaged that on a case-by-case basis lesser amounts of event-specific data are needed for the risk assessment.

While addressing question two of the mandate, the EFSA GMO Panel compared the hazards associated with plants produced by cisgenesis and intragenesis with those obtained by either conventional plant breeding techniques or by transgenesis. The Panel concludes that similar hazards can be associated with cisgenic and conventionally bred plants, while novel hazards can be associated with intragenic and transgenic plants. The Panel is of the opinion that all of these breeding methods can produce variable frequencies and severities of unintended effects. The frequency of unintended changes may differ between breeding techniques and their occurrence cannot be predicted and needs to be assessed case by case. Independent of the breeding method, undesirable phenotypes are generally removed during selection and testing programmes by breeders. The risks to human and animal health and the environment will depend on exposure factors such as the extent to which the plant is cultivated and consumed.

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BACKGROUND AS PROVIDED BY THE EUROPEAN COMMISSION

New breeding and genetic modification techniques have evolved at a rapid pace since the introduction of the legislation in 1990 with the result that in some instances it is unclear whether they give rise to GMOs pursuant to EU Legislation. This is especially relevant for plant breeding as some of these breeding and genetic modification techniques have been subject to field trials in the EU and a number of them are now approaching commercialisation.

At the request of the Competent Authorities (CA) under Directive 2001/18/EC, a New Techniques Working Group (NTWG) was established in October 2007 to analyse a non-exhaustive list of techniques for which it is unclear whether they would result in a genetically modified organism or a genetically modified micro-organism as defined under Directive 2001/18/EC or Directive 2009/41/EC respectively.

An initial list of eight techniques was proposed by the CA for consideration by the NTWG⁴. At the time of requesting this opinion the most recent version of the report has been provided for information.

The Terms of Reference as endorsed by the CA state that “the findings of the WG may be referred to EFSA for opinion”. In addition, the Netherlands has transmitted recently a request to the Commission concerning the safety of cisgenesis, one of the techniques addressed by the Working Group.

BACKGROUND AS PROVIDED BY EFSA

Following a request of the European Commission (DG SANCO Ares(2011)201516 – 23/02/2011), EFSA set up a working group of the EFSA GMO Panel on new techniques during April 2011. As requested by the European Commission the EFSA GMO Panel working group initiated with cisgenesis and intragenesis. The EFSA GMO Panel working group considered the definitions of cisgenesis and intragenesis as defined by the New Techniques Working Group of the Member States’ experts (see above).

TERMS OF REFERENCE AS PROVIDED BY THE EUROPEAN COMMISSION

Against this background, the Commission would like to ask EFSA to address the following considerations in separate opinions per technique or for groups of techniques as appropriate:

1. Determine whether there is a need for new guidance or whether the existing guidance on risk assessment should be updated or further elaborated, in anticipation of the placing of products on the market through the application of the listed techniques.
2. What are the risks in terms of impact on humans, animals and the environment that the eight techniques listed could pose, irrespective of whether or not they fall under the GMO legislation? This latter request should consider the most recent scientific literature and knowledge of plant breeding experts and compare plants obtained by these new techniques with plants obtained by conventional plant breeding techniques and secondly with plants obtained with currently used genetic modification techniques.

⁴ The following techniques were proposed by the Competent Authorities for consideration in a first stage by the NTWG: (1) zinc finger nuclease technology (ZFN), comprising ZFN-1, ZFN-2 and ZFN-3 as defined in the current draft of the report; (2) oligonucleotide-directed mutagenesis (ODM); (3) cisgenesis (comprising cisgenesis and intragenesis); (4) RNA-dependent DNA methylation via RNAi/siRNA; (5) grafting; (6) reverse breeding; (7) agro-infiltration; (8) synthetic biology.

ASSESSMENT

1. Introduction

In dealing with the requests from the European Commission (EC) the EFSA GMO Panel has, in this opinion, focused initially on the hazards associated with cisgenic and intragenic plants compared with those developed by conventional breeding or currently used genetic modification techniques. As requested by the EC, the Panel considered the most recent and relevant scientific literature together with the expertise and experience of plant breeding experts. The Panel then considered the applicability of its current guidance documents on the risk assessment of genetically modified (GM) plants to the assessment of plants developed by cisgenesis and intragenesis. Cisgenesis and intragenesis were defined by a working group of EU Member States' experts on new techniques as follows:

“Cisgenesis is the genetic modification of a recipient organism with a gene from a crossable – sexually compatible – organism (same species or closely related species). This gene includes its introns and is flanked by its native promoter and terminator in the normal sense orientation.

Cisgenic plants can harbour one or more cisgenes, but they do not contain any parts of transgenes or inserted foreign sequences. To produce cisgenic plants any suitable technique used for production of transgenic organisms may be used. Genes must be isolated, cloned or synthesized and transferred back into a recipient where stably integrated and expressed.

*Sometimes the term cisgenesis is also used to describe an Agrobacterium-mediated transfer of a gene from a crossable – sexually compatible – plant where T-DNA borders may remain in the resulting organism after transformation. This is referred further in the text as **cisgenesis with T-DNA borders**.*

Intragenesis is a genetic modification of a recipient organism that leads to a combination of different gene fragments from donor organism(s) of the same or a sexually compatible species as the recipient. These may be arranged in a sense or antisense orientation compared to their orientation in the donor organism. Intragenesis involves the insertion of a reorganised, full or partial coding region of a gene frequently combined with another promoter and/or terminator from a gene of the same species or a crossable species.”

With regard to comparisons between the safety of cisgenic/intragenic plants and those developed by conventional breeding and genetic modification techniques currently used, the EFSA GMO Panel has used the following approach:

- i) An assessment of conventional breeding approaches as the baseline for all comparisons. This includes a description of the sources of genes used by breeders and of the conventional breeding approaches most closely related to cisgenic and intragenic breeding;
- ii) A comparison of the sources of hazards arising from conventional breeding and cisgenic, intragenic breeding and genetic modification techniques currently used (i.e. transgenesis).

With regard to the applicability of current EFSA guidance documents for the risk assessment of transgenic plants to the assessment of plants developed by cisgenesis and intragenesis, the EFSA GMO Panel has evaluated all components of the molecular characterisation, food/feed and environmental safety evaluations with regard to the question whether the existing guidance on risk assessment should be updated or further elaborated.

2. Conventional plant breeding

Within the context of this document, conventional plant breeding is defined as methods used by plant breeders for the improvement of commercial varieties and where the resulting plants/varieties are not covered by the definitions of genetic modification in Directive 2001/18/EC.

Plant breeding for the improvement of commercial varieties includes two principal steps: (A) introduction of genetic variation; and (B) selection for the presence of desired traits and absence of undesired traits among the plants available from (or generated in) step A. For any given plant species the genes and alleles required for the development of new, advanced varieties may already exist within the current gene pool of commercial lines. In other cases the plant breeder needs to access genes and alleles from a wider gene pool to obtain the required traits of interest, e.g. resistance to evolving pests and pathogens. Methods used to introduce genetic variation are diverse, ranging from approaches to manipulate whole genomes (polyploidisation), to introduce whole or partial genomes from wild relatives of cultivated plants (wide crosses, introgression and translocation breeding), or to produce mutations in the genome using specific mutagenic agents (chemicals, radiation, chemical mutagens). In general, all breeding approaches require subsequent selection steps to maximise the benefits and minimise undesirable consequences. Selection techniques range from phenotyping for agronomic performance to more sophisticated techniques such as marker-assisted selection. Before commercialisation, all new varieties have to be shown to be Distinct, Uniform and Stable (DUS), for National Listing, Plant Breeders' Rights and Multiplication (Certification).

2.1. Sources of genes – the plant breeders' gene pool

The sources of genes available for conventional plant breeding are referred to as the “breeders' gene pool”. Breeders distinguish between primary, secondary and tertiary gene pools. Each primary gene pool comprises one cultivated species together with other taxonomic species with which it can interbreed freely. The secondary gene pool includes species that can be cross-bred only with difficulty with a member of the primary gene pool but which produce at least some fertile hybrids. The tertiary gene pool comprises those species that are more distantly related to a member of the primary gene pool, but which can be cross-bred only using advanced techniques such as embryo rescue, induced polyploidy and bridge crosses. Breeders are continually expanding the tertiary gene pool and will continue to do so in the future.

In addition to the nuclear genome, plants also possess organelle genomes of the plastid and mitochondria. Organelle genomes are inherited either uniparentally (usually via the gametic cytoplasm of the maternal parent) or biparentally, and lack the processes of random assortment and segregation associated with meiosis and zygotic fertilisation of gametes. Thus, during conventional breeding, organelle genomes are inherited largely unaltered from one generation to another and it is currently not possible to breed for individual organellar genes. Plastid transformation in some plant species is well established and, although there are no current commercial applications, it is feasible to use genetic engineering techniques to transfer a plastid gene from one cultivar or line to another.

As conventional plant breeding can make use of the primary, secondary and tertiary gene pools, the EFSA GMO Panel considers that genes derived from these gene pools should be regarded as cisgenes. The Panel also considers the transfer of organellar genes between plants within the breeders' gene pool where the gene is integrated in the same plant organelle from which it was derived (e.g. plastid) to fall within the concept of cisgenesis.

2.2. Introduction of genetic variation

Plants are cross-bred to introduce traits/genes from one variety or line into a new genetic background. Wild relatives are often used as sources of desirable traits lacking in the plant species in question, e.g. disease and pest resistance. Sometimes wild relatives hybridise naturally with the cultivated species; this is the most traditional way of introducing genetic variation. On other occasions the breeder has to overcome barriers to hybridisation using special techniques such as bridge crosses, embryo rescue and protoplast fusion (Evans, 1983; reviewed by Fedak, 1999; Lynch et al., 1993; Mathias et al., 1990; Stewart, 1981; Van Eijk et al., 1991).

Once the new combination of genetic material has been produced, the introgression of desired genes/alleles into the cultivated species can be completed by recurrent backcross breeding to the

cultivated species. Selection for the desired trait is balanced against any undesirable characteristics and phenotypes from the wild relative. Such selection is necessary for all breeding approaches irrespective of technologies used. If the genomes of the wild and cultivated species are (structurally) similar, the introgressed gene(s) will integrate into the cultivated species through normal sexual reproduction (i.e. homologous recombination). This means that introgression will always transfer (relatively) small regions of the wild relatives' chromosomes rather than individual genes. If the genes for undesirable traits are tightly linked to those being introgressed, linkage drag will lengthen the breeding process as more backcrosses will be required to break the linkage. In contrast, cisgenesis transfers individual genes in one step avoiding linkage drag (Schouten et al., 2006).

Conventional breeding includes a wide range of breeding techniques (see section 2); an overview of conventional plant breeding techniques is provided by Van der Wiel et al. (2010). The EFSA GMO Panel does not consider them all to be relevant for a comparison with cisgenesis/intragenesis. The following conventional breeding techniques are considered most relevant for the comparison with cisgenic and intragenic plants:

- *Sexual crosses within species*: The most frequently used method of introducing new variation in plant breeding involves the production of viable offspring through the crossing of closely related parental lines selected on the basis of the attributes the breeder wishes to combine. Taking maize as an example, commonly two populations of almost homozygous breeding lines with desired characteristics are developed by backcrossing to the original parents and self-fertilisation until the homozygosity of the population exceeds a certain level, usually 90 % or more. Once completed both populations are crossed to combine the desired traits in the F1 seed. The divergence between the parent lines promotes improved growth and yield characteristics through the phenomenon of heterosis (“hybrid vigour”), whilst the homozygosity of the parent lines ensures a phenotypically uniform F1 generation. Self-fertilisation is avoided in the production of F1 seed, e.g. by deactivating or removing male flowers from one population.
- *Bridge crosses*: Bridge crosses make it possible to exploit new sources of traits lacking from directly cross-compatible species (Khrustaleva and Kik, 2000; reviewed by van der Wiel et al., 2010; Van Eijk et al., 1991). When a direct cross between two species is not possible, an intermediate crossing with a third species, which is compatible with both species, can be used to bridge the crossing barrier. As an example, genes in *Solanum bulbocastanum* which confer resistance to potato late blight caused by *Phytophthora infestans* have been transferred by “double-bridge” crosses between four different *Solanum* species $\{[S. acaule \times S. bulbocastanum] \times S. phureja\} \times S. tuberosum$. In this case, tetraploid *S. acaule* (wild potato) was crossed with diploid *S. bulbocastanum* (ornamental nightshade) and the resulting triploid hybrid was doubled to a hexaploid using the mitotic inhibitor colchicine; the hexaploid was subsequently crossed with diploid *S. phureja* (nightshade). In this way, a tetraploid hybrid was obtained that could be crossed to potato (*S. tuberosum*) to introgress genes from *S. bulbocastanum* (Hermsen and Ramanna, 1973).
- *Wide crosses using embryo rescue*: Interspecific incompatibility in plants can occur for many reasons, usually leading to embryo abortion (Reed, 2005). In plant breeding, wide hybridisation crosses can result in small shrunken seeds, which indicates that fertilisation has occurred, but the seed fails to develop. Embryo rescue deploys *in vitro* culture techniques to assist in the development of plant embryos that might not otherwise survive, to become viable plants (Cisneros and Tel-Zur, 2010; Mathias et al., 1990; Poysa, 1990; Stewart, 1981). Embryo rescue plays an important role in current plant breeding, and allows the production of interspecific and intergeneric food and ornamental plant hybrids. The immature or weak embryos are nurtured in culture, facilitating survival. The most widely used embryo rescue procedure is embryo culture – used to create interspecific and intergeneric crosses that would normally produce seeds which are aborted (Miyajima, 2006).

- *Translocation breeding*: When the wild relative's chromosome is distantly related to its equivalent in the cultivated species, ordinary chromosome pairing and genetic recombination during sexual reproduction cannot be relied upon to transfer the desired gene(s) from the chromosome of wild to cultivated plant. Special techniques collectively referred to as chromosome manipulation must be used to incorporate the desired gene(s), and these are confined to allopolyploids tolerant of chromosome addition and substitution (Chu and Zhang, 1985; Islam et al., 1981; Rayburn and Carver, 1988). Radiation, such as X-rays and gamma rays, is used to break chromosomes more or less randomly. If a translocated fragment contains the desired gene but no other genes with undesirable effects, and no undesirable position effects result from genes in new chromosome locations, the new recombinant chromosome can be incorporated in the genome of the cultivated species. There are examples of the use of translocations in tobacco (transfer of tobacco mosaic virus resistance from *Nicotiana glutinosa* to *N. tabacum*) and wheat (rust resistances from rye and wild diploids) (Ko et al., 2002; Lewis et al., 2005).
- *Somatic hybridisation (protoplast fusion)*: Somatic hybridisation is excluded from the GMO legislation when it involves the fusion of plant cells of organisms which can exchange genetic material through conventional breeding methods. Such *in vitro* cell fusion techniques can increase the efficiency of generation of hybrids that can be developed by sexual crossing only with difficulty (Evans et al., 1983; Glimelius et al., 1991; Liu et al., 2005). The fusion is induced chemically (e.g. polyethylene glycol) or electrically. The technique has been applied to many species, e.g. carrot, tobacco, rapeseed and potato. In potato a polyploid fusion product between tetraploid *S. tuberosum* and wild diploid *S. brevidens* has been developed to obtain virus resistance. Somatic hybridisation can be used to combine specific nuclear and organellar (plastid and mitochondrial) genomes in an efficient manner, avoiding lengthy backcrossing.
- *Somaclonal variation*: The term describes the variation seen in plants that have been produced by plant tissue culture. This variation is particularly common in plants regenerated via a callus phase. Chromosomal rearrangements are an important source of this variation (see section 4.2 for details of underpinning mechanism). Somaclonal variation has been observed in many plant species and is an alternative way to create variants and expand the germplasm pool (Evans, 1989; Larkin and Scowcroft, 1981).

3. Introduction of genetic variation by cisgenesis, intragenesis and transgenesis

Cisgenesis and intragenesis make use of the same transformation techniques as transgenesis. Commonly used methods will result in random integration of the gene in the plant genome although in the future site-directed integration might become more widely available.

Cisgenesis: Specific alleles/genes (referred to in the text as cisgenes) present in the breeders' gene pool are introduced, without any change to the DNA sequence, into new varieties without the accompanying linkage drag (co-transfer of DNA sequences that are linked to the gene of interest) which occurs in conventional breeding. Linkage drag is particularly problematic in the conventional breeding of plants with a long generation time (e.g. fruit tree species) or for outbreeding plant species with complex genetics (polyploidy, vegetatively propagated), e.g. potato or banana. Cisgenesis can thus accelerate the breeding of species with long reproduction cycles (e.g. trees).

Intragenesis: Like cisgenic plants, intragenic plants contain genetic elements from the breeders' gene pool. However, in intragenesis desired traits can be created by recombining genetic elements such as promoters, coding sequences and terminators of different genes within the gene pool (the gene produced by combining different elements is referred to in the text as an intragene). Intragenesis therefore offers considerably more options for modifying gene expression and for trait development. Intragenesis can also include hairpin gene silencing approaches (Nielsen, 2003; Rommens, 2007; Rommens et al., 2007; Rommens et al., 2008).

Transgenesis: Recombinant nucleic acid techniques are used to form new combinations of genetic material from outside the breeders' gene pool which is incorporated into the plant. Transgenic plants can contain genetic elements, e.g. coding and regulatory sequences, from any organism (eukaryotic, prokaryotic) as well as novel sequences synthesised *de novo*.

4. Hazard identification addressing question two of the mandate: identification of characteristics with the potential to cause adverse effects

When considering hazards related to cisgenic and intragenic plants compared with transgenic and conventionally bred plants the major considerations by the EFSA GMO Panel include the source of the DNA and the safety of gene products; alterations to the host genome at the insertion site and elsewhere; the potential presence of non-plant sequences in the insert; the expression of the trait and its potential wider implications.

4.1. Source of genes and safety of gene products

As conventional plant breeding can make use of the primary, secondary and tertiary gene pools the EFSA GMO Panel considers that genes derived from these gene pools can be used for cisgenesis and intragenesis. With regard to the sourcing of cisgenes several examples of types of cisgenic plants can be envisaged:

- the donor plant (e.g. variety, landrace, wild relative) has a history of cultivation and consumption by humans;

Researchers are currently exploring the possibility to transfer resistance genes from cooking banana to dessert banana, both having a history of consumption, to protect against *Fusarium* wilt (*Fusarium oxysporum* f. sp. *Cubense*). As banana is practically sterile it is almost impossible to introgress resistance genes by conventional crossing and the cisgenic approach can provide a solution. In the 1960s a new race of *Fusarium* made production of the 'Gros Michel' dessert banana variety impossible and 'Gros Michel' was replaced by 'Cavendish'. Since then *Fusarium* has evolved, causing disease in 'Cavendish'. Currently the only option for continued long-term production of banana is to avoid spread of the disease and the replacement of the susceptible variety with a resistant one. No suitable replacement has been identified for dessert banana until now

(www.nt.gov.au/d/Content/File/p/Plant_Pest/786.pdf).

- the donor plant has no history of consumption by humans, but has been used in conventional breeding;

Early in the 20th century the potential of the wild Mexican hexaploid potato *Solanum demissum* was recognised as conferring exceptionally strong late blight resistance, and hybrids with potato *S. tuberosum* were generated. Subsequently, 11 major dominant resistance (*R*) genes were identified, and introgressed into potato varieties by interspecific hybridisation and backcrosses with cultivated potato (Malcolmson, 1969; Malcolmson and Black, 1966; Muller and Black, 1952). Although the 11 genes, separately and in combinations, failed to provide durable resistance, many of them are present in widely grown varieties (e.g. Pentland Dell). Other wild species used to breed pest and disease resistance in commercial potato varieties include *S. vernei*, *S. microdontum*, *S. multidissectum*, *S. commersonii*, *S. maglia* and *S. acaule* (Bradshaw, 2009). These wild species have a history of use in providing a source of genes (not only resistance genes) to be introgressed into commercial potato varieties which now have a history of safe consumption. However, the wild species themselves are not part of the food chain.

- the donor plant has not been exploited yet for variety development, but there is knowledge of the gene family in terms of the structure and functions of the proteins they encode;

Breeding for late blight resistance in potato relies heavily on the deployment of major resistance genes against the causal organism *Phytophthora infestans* (so called *Rpi* genes). Recent germplasm screens are revealing a wealth of *Rpi* genes in wild *Solanum* species and the challenge now is how to select, judiciously combine and deploy sets of *Rpi* genes that can confer durable late blight resistance in modern potato varieties. A wild diploid potato species, *S. bulbocastanum*, is highly resistant to all known races of *P. infestans*, even under intense disease pressure (Helgeson et al., 1998). However, *S. bulbocastanum* resistance sources have not been widely exploited yet for variety development, mainly due to crossing barriers with cultivated potato (Helgeson et al., 1998; Hermesen and Ramanna, 1973). However, somatic hybrids between *S. bulbocastanum* and *S. tuberosum* have been developed with the long-term goal of capturing resistance for use in potato varieties. Sexual backcrosses of those somatic hybrids were easily obtained and late blight resistance was successfully transferred to their progeny.

Plants are known to contain hundreds of *R* genes (Sanseverino et al., 2010), which have thus been commonly consumed. The molecular characterisation of numerous *R* genes from diverse plant species conferring resistance to bacteria, fungi, viruses, nematodes and insects has revealed features characteristic of *R* genes likely to form the basis of *R* gene specificity and rapid evolution. The majority of *R* genes are members of tightly linked multigene families.

- None of the above

In this case the donor plant has not been exploited yet for variety development and gene family and the mode of function of the protein is not well established.

With regard to conventional breeding, donor plants falling under all of the above scenarios can be used for plant improvement. Indeed, with time the breeders' gene pool will become even more extensive through the use of more exotic species from germplasm collections. The concept of history of safe use was not developed with respect to plant breeding *per se* but for the assessment of imported food. Indeed, varieties of some species may only be on the commercial market for 2 to 3 years before they are superseded by superior lines. In these cases any "history of safe use" would have to be associated with the plant species in a generic sense, e.g. with wheat, maize potato, etc., rather than with individually derived varieties or genes.

Cisgenesis and intragenesis do not introduce the range of other genes and sequences that can be associated with linkage drag in conventional breeding and thus avoid introducing the unwanted traits and hazards associated with these other genes/sequences. With respect to the introduced genes, the hazards arising from the use of a related plant-derived gene by cisgenesis are similar to those from conventional plant breeding, as similar traits are expressed by the gene. However when a related plant-derived gene is used in intragenesis some new combinations of genetic elements may arise that are not found in cisgenic and conventionally bred plants and these may present novel traits with novel hazards. Hazards can be identified which are specific for transgenic plants as the transgenes and their gene products can be obtained from any source including non-plant.

4.2. Alterations to the genome

During the development of cisgenic, intragenic and transgenic plants alterations to the genome can be expected to occur over and above the introduction of the inserted gene(s). These alterations can be caused by various processes and mechanisms. Where undesirable changes in the genome occur in conventional breeding or in the production of transgenic, cisgenic or intragenic plants they might be removed by backcrossing. This will depend on the reproductive biology of the species and the commercial practices used for breeding.

Mechanisms of DNA integration: Cisgenesis and intragenesis make use of the same transformation techniques as transgenesis. *Agrobacterium*-mediated transformation and "direct gene transfer" methods have been developed for the stable transformation of plants (reviewed by Vain, 2007). Direct gene transfer methods use external physical or chemical factors for the introduction of naked DNA

into the plant cell, while *Agrobacterium*-mediated transformation uses the natural ability of the bacterium to transfer a T-DNA to the plant cell. In general, integration patterns obtained by *Agrobacterium*-mediated transformation are regarded as more precise and less complex when compared to the integration patterns of DNA delivered to the plant cell by means of direct gene transfer methods (Cheng et al., 1997; Dai et al., 2001; Kohli et al., 2003; Travella et al., 2005).

Integration of cisgenes, intragenes and transgenes into the plant genome requires chromosome breaks and natural DNA repair mechanisms. Independent of the methodology, DNA is integrated by means of illegitimate recombination into breaks (reviewed by Edlinger and Schlogelhofer, 2011; Filipenko et al., 2009; Tzfira et al., 2004). Double-stranded breaks can arise in all tissues (e.g. by excision of transposable elements, irradiation, reactive oxygen species and meiotic recombination). In somatic tissues double-stranded breaks in DNA are mostly repaired via the non-homologous end joining pathway (Gorbunova and Levy, 1999; Puchta, 2005; Waterworth et al., 2011; Wicker et al., 2010).

Sites of DNA integration: Since transgenes, cisgenes and intragenes integrate into the plant by illegitimate recombination mechanisms, there is no preference for similar nucleotide sequences in the genome for the integration process (Alonso et al., 2003). However, transgenes and, in particular, T-DNA-linked transgenes do show a high preference for gene-rich regions (Cellini et al., 2004 and references therein). In this respect, the integration of transgenes (and again by inference cisgenic and intragenic DNA) is similar to naturally occurring recombination processes in plant genomes. It is well established that for many plant species large differences in natural recombination rates occur over a great portion of the genome. Genes *per se* and low-copy DNA are hotspots for natural recombination (Conner and Jacobs, 1999). In wheat, for example, genes are grouped into close clusters, and gene-rich regions are hotspots for recombination (Gill et al., 1996).

Genome disruptions, deletions and rearrangements: The potential for “random” changes to the genome caused by the insertion event is not limited to transgenesis, cisgenesis and intragenesis. Insertional mutagenesis is known to occur naturally through the random movement of the numerous mobile genetic elements such as transposons and retrotransposons, which are present in all plant genomes with varying prevalence. The movement of transposons and retrotransposons may affect genes in many ways. Their insertion within genes may result in gene disruption, and this has been used to generate mutant collections. Indeed, the skin colour in white grapes, an important trait for grape, berry and wine quality, has been shown to be the consequence of a retrotransposon insertion in the promoter of a *Myb*-related gene that regulates anthocyanin biosynthesis (Kobayashi et al., 2004). This insertional mutation is present in most white grape varieties (Walker et al., 2007). In addition to gene disruption, transposons can also induce chromosomal breaks, which can result in inversions, deletions and duplications (Bennetzen, 2002; English et al., 1995; Kloeckenergruissem and Freeling, 1995; Rubin and Levy, 1997; Walker et al., 1995). The types of allelic variants range from single nucleotide polymorphisms (SNPs), and insertions/deletions, to large structural changes of several kilobases of DNA fragments (Fu and Dooner, 2002).

It is well known that changes to the genome occur during conventional breeding also because the DNA damage repair system is more error prone in plants than in other species. The repair process is associated with rearrangements that can lead to DNA deletions, insertions (including “filler” DNA, i.e. short scrambled sequences new to the plant), duplications, inversions and translocations (Goettel and Messing, 2009; Gorbunova and Levy, 1999; Messing and Bennetzen, 2008; Puchta, 2005; Salomon and Puchta, 1998). As the same mechanisms for DNA repair are involved in the integration of recombinant DNA, such changes can occur at the insertion site and have been observed on many occasions in transgenic plants (Castle et al., 1993; Forsbach et al., 2003; Gheysen et al., 1987; Laufs et al., 1999; Nacry et al., 1998; Ohba et al., 1995; Tax and Vernon, 2001). The same type of change is therefore also expected in cisgenic and intragenic plants as in conventional breeding.

Somaclonal variation: This arises from maintaining plants in tissue culture, particularly where a callus phase is involved. Somaclonal variation can cause significant changes to the genome (Labra et al.,

2004) and is used as a source of variation in conventional plant breeding (Kaepler and Phillips, 1993; Kaepler et al., 1998; Sunderland, 1973). Similarly to conventional breeding, the effects of somaclonal variation will be present in trans-, cis- and intragenic plants. Several mechanisms have been implicated in somaclonal variation:

- chromosomal rearrangements and changes in chromosome number (Kaepler et al., 1998);
- mutations are frequent among tissue culture regenerants, and single-base changes or very small insertions/deletions are the basis of these changes (Brettell et al., 1986; Dennis et al., 1987; Jiang et al., 2011);
- variation in DNA methylation has been hypothesised as a mechanism explaining tissue culture-induced mutagenesis due to the high frequency of quantitative phenotypic variation, activation of transposable elements, heterochromatin-induced chromosome breakage events, and the high frequency of sequence changes. Methylation patterns were first reported to vary among regenerated plants and their progeny in rice (Brown et al., 1990) and maize (Brown et al., 1991). In some cases changes in the genome are caused by transposable element and retrotransposon activity (Hirochika et al., 1996; Peschke and Phillips, 1991; Peschke et al., 1987; Robertson, 1978; Sabot et al., 2011). This supports the argument that de-repression of epigenetically silenced sequences can be induced by the culture process.

Creation of novel open reading frames (ORFs): An ORF is defined as any nucleotide sequence that consists of a string of codons that is uninterrupted by the presence of a stop codon in the same reading frame (EFSA, 2011). Therefore, introduction of a new fragment will result in the creation of new ORFs. The formation of new ORFs will occur at random for all processes which involve the insertion of DNA fragments into plant genomes. This includes conventional breeding, cisgenesis, intragenesis and transgenesis and in all cases could give rise to new proteins. However, the presence of an ORF does not necessarily indicate a hazard. ORFs created in transgenic plants can differ from those created by conventional breeding and cisgenesis in those cases where exogenous, foreign DNA is involved leading to the formation of potentially new sequence combinations which would normally not arise in conventional breeding or cisgenesis. Similarly, due to the fact that host sequences can be reconfigured using intragenesis, potentially new sequence combinations could arise in ORFs. It should be noted that in case of cisgenesis or intragenesis with T-DNA borders some of the ORFs could only arise due to insertion of filler sequences and similar sequences can be present in the plant genome (Lusser et al., 2011). These items are further discussed in section 4.3.

In summary, unintentional changes to the genome can arise during transgenesis, intragenesis and cisgenesis and result in a safety issue. However, the same mechanisms and types of unintentional genome changes occur during conventional breeding as it is well known that the plant genome is not a fixed entity (see Casacuberta and Puigdomènech, 2000). The high degree of plasticity in plant structure and physiology probably allows plants to tolerate large quantities of genomic rearrangements, which has been a driving force in plant evolution. Nucleotide sequence variation exists widely within a species as exemplified by the maize genome which has an especially high level of DNA sequence polymorphism, approximately an order of magnitude higher than that in humans (Bhatramakki et al., 2002; Buckler and Thornsberry, 2002; Ching et al., 2002; Sunyaev et al., 2000). Recently Morgante et al. (2007) introduced the concept of a “pan genome” following the observation that a single genome sequence might not reflect the entire genomic complement of a species. The pangenome concept defines core genomic features that are common to all individuals in the species and a dispensable genome composed of partially shared and/or non-shared DNA sequence elements. This concept is further supported by evidence indicating that structural variation (presence or absence of specific sequences and copy number variation) can lead to variation in the genome content of individuals within a species (Swanson-Wagner et al., 2010; Wang and Dooner, 2006).

The EFSA GMO Panel found that all of these breeding methods can produce variable frequencies and severities of unintended effects. The frequency of unintended changes may differ between breeding techniques and their occurrence cannot be predicted and needs to be assessed case by case.

4.3. Presence of non-plant sequences in the insert

In some cases, as with transgenesis, short sequences not originating from the donor plant might be inserted into the recipient plant together with the cisgene or intragene. These sequences might include for example multiple cloning sites, or T-DNA borders in the case of *Agrobacterium*-mediated transformation. These issues are specifically linked to the transformation method used and are not of relevance for conventionally bred plants.

T-DNA border sequences: In *Agrobacterium*-mediated transformation the DNA fragment expected to be inserted into the plant is called T-DNA (transfer-DNA). The T-DNA is flanked by the left (LB) and right (RB) border repeats. A T-DNA border repeat consists of a maximum of 25 base pairs (Gheysen et al., 1998). The T-strand transferred from *Agrobacterium* to the plant normally contains three nucleotides from the RB repeat and up to 22 nucleotides from the LB repeat at the 3' end. Random deletions at one or both ends of the T-DNA can occur during integration, resulting in the absence of these border sequences in the cis-, intra- or transgenic plant (Brunaud et al., 2002; Deroles and Gardner, 1988; Kim et al., 2003; Krysan et al., 2002; Meza et al., 2002; Windels et al., 2003).

Database similarity searches indicate that sequences similar to the 22-bp LB repeat can be found in plants (Rommens, 2004). In some cases they may be remnants from a natural transformation event by *A. tumefaciens* or *A. rhizogenes* (Intrieri and Buiatti, 2001). Therefore, specific vectors have been constructed for cisgenic/intragenic approaches which use DNA sequences originating from the same plant species or related species to insert the target genes to circumvent issues arising from the use of bacterial border sequences and their inclusion in the transgenic plant. This approach is termed the P(plant)-DNA approach (Conner et al., 2007; Rommens et al., 2004). A P-DNA region isolated from potato was shown effective in *Agrobacterium*-mediated transformation of potato. The general presence of such P-DNAs within the genomes of different plant species remains to be established.

As the border repeat sequences (T-DNA and P-DNA) delineate the DNA of interest, they will be fused to plant sequences and will create novel open reading frames. However, there are a limited number of nucleotides of the T-DNA border repeats (usually 22-bp from the LB repeat) that can encode for a maximum of eight amino acids (Jacobsen and Schaart, 2009).

Vector backbone sequences: Vector backbone sequences may be transferred to the plant when using the *Agrobacterium*-mediated transformation (Kim and Lee, 2007; Kononov et al., 1997; Meza et al., 2002; Permyakova et al., 2009; Petti et al., 2009; Podevin et al., 2006; Ramanathan and Veluthambi, 1995; Smith et al., 2001; van der Graaff et al., 1996; Wenck et al., 1997; Wolters et al., 1998). This is often caused by transfer of longer T-DNAs due to read-through of the LB repeat, or by the initiation of transfer at the LB instead of RB repeat. As a result, part of the vector backbone remains attached to the border, and may be co-inserted into the plant genome. By definition, cisgenic and intragenic plants must not contain vector backbone sequences of bacterial origin (Jacobsen and Schouten, 2009).

Selectable marker genes: Transgenic plants are usually selected using marker genes (e.g. for herbicide tolerance) that are introduced into the plant together with the donor gene(s). Marker genes are not usually derived from the recipient species (breeders' gene pool) and in such cases cannot be present in cisgenic/intragenic plants. Exemptions could include the use of genes from the breeders' gene pool (Rosellini, 2011), which themselves can confer herbicide resistance or other traits useful for plant selection. Marker genes can be eliminated from the final plant by several techniques and with different outcomes (Darbani et al., 2007). For example:

- Two independent DNA insertions at unlinked positions in the genome can be separated via segregation by conventional breeding (An, 1987; Daley et al., 1998; De Buck et al., 1998; de

Framond et al., 1986; Huang et al., 2004; Komari et al., 1996; Lu et al., 2001; Xing et al., 2000).

- Selectable markers can be recombined out by using site-specific recombinase *in trans* (Dale and Ow, 1991; Gilbertson, 2003; Hoa et al., 2002; Russell et al., 1992; Srivastava and Ow, 2004; Woo et al., 2009). This approach has been used in the creation of cisgenic apple (Vanblaere et al., 2011). This will, however, leave behind a recombination site. In the case of gene stacking, the presence of multiple recombination sites may cause inter- and intra-chromosomal rearrangements. Marker removal systems have also been developed in which the marker gene is present on a transposable element in the T-DNA, called the multi-auto-transformation vector (MAT) (Ebinuma et al., 2005; Ebinuma et al., 1997; Sugati et al., 2000; Sugati et al., 1999). Subsequent transposon excision allows the development of plants without a selectable marker.

4.4. Modification of gene expression

Given the known plasticity of the plant genome, conventional breeding is expected to result in changes in genome-wide gene expression patterns in the progeny compared with the parental lines (Birchler et al., 2010 and references therein). Indeed, such changes are desired for improved agronomic performance. Gene expression profiles will be further modulated through genotype × environment interactions, which is also the case for cisgenes, intragenes and transgenes. Most transcript, protein and metabolite levels are influenced by multiple genetic and environmental (biotic and abiotic) factors (HESI, 2010), indicating wide acclimation and compensatory capabilities of plants. Depending on each particular case some factors play a more prominent role than the others. The expression profiles of transgenic varieties compared to their appropriate comparators have often been shown to be less variable than those between conventional varieties (Batista et al., 2008; Kogel et al., 2010; Lehesranta et al., 2005; Ruebelt et al., 2006). Indeed, there is a growing body of literature derived from the analysis of thousands of transcripts, proteins and metabolites that variation in expression most often falls within the range of natural variation found in ecotypes, landraces and commercial varieties (HESI, 2010 and references therein). Alteration of gene expression at the mRNA level usually leads to altered levels of proteins and metabolites. Novel proteins and metabolites or increased levels of endogenous proteins and metabolites might raise safety issues in both natural variants and plants generated by any breeding technology.

Position effects of the insert: The integration of transgenes, cisgenes and intragenes in plant genomes can take place virtually at any place in the genome (Chyi et al., 1986; Filipecki and Malepszy, 2006; Wallroth et al., 1986), as is also the case for transposons and retrotransposons. This can lead to so-called position effects, which describe the variation in the expression of identical genes inserted into different regions of a genome. The random integration of the cis/intra/transgene in plant genomes can influence the expression of genes or affect the functionality of regulatory elements around the site of integration (Schaart and Visser, 2009). The inserted DNA may also have an enhancing or silencing effect on the expression of genes of the recipient (Tani et al., 2004).

Whilst numerous factors can influence the level of cisgene, intragene and transgene expression in the plant, the three most important factors are the characteristics of the genetic construct introduced, the number of copies inserted (reviewed by Dietz-Pfeilstetter, 2010) and the site of insertion. Integration may occur into regions with generally high or low transcriptional activity, and the presence of nearby regulatory sequences such as transcriptional enhancers and inhibitors may influence expression (Kang et al., 2007; Kim and Grierson, 2005; Matzke and Matzke, 1998; Meyer, 2000; Zheng et al., 2008). Gene expression can also be negatively influenced by epigenetic silencing phenomena (Weil and Martienssen, 2008), either post-transcriptional gene silencing (PTGS or RNAi) or transcriptional gene silencing (TGS). Homology-based silencing may take place if different coding sequences under the same promoter are combined by sexual crossing, as may be the case when events are stacked (Daxinger et al., 2008; Fagard and Vaucheret, 2000; Matzke et al., 1993). Newly introduced genes may also cause co-suppression of homologous endogenous genes (Napoli et al., 1990).

Promoter functionality: The regulatory sequences linked to the coding region of the gene of interest are critical for its expression pattern (temporal, spatial, response to internal and external factors). The promoter (its core, proximal and distal elements) is the major factor determining the level of gene expression, although the terminal and internal regions (introns) can also have an influence. The promoter consists of an array of regulatory elements which impact on gene expression in a concerted manner (Abeel et al., 2009; Schaart and Visser, 2009). The most proximal core region provides the basal expression, which is modulated by the more distant regulatory elements. These elements are generally located within a DNA fragment of 500–1000 base pairs directly upstream of the coding sequence (Barta et al., 2005), but promoters of plant genes can have regulatory elements that are positioned several kilobases away from the transcriptional start site (Goni et al., 2007; Szankowski et al., 2009) or located downstream or within the transcribed region. The promoter can contain tens or even hundreds of overlapping domains that may be influenced by various internal and external factors. The prediction of the gene expression levels is therefore currently not possible on theoretical grounds.

In cisgenesis, the transferred genes are derived from sexually crossable species (alleles of endogenous genes), and they will always be flanked by their native promoters. Whilst it might be expected that the use of a native promoter is more likely to result in similar levels of expression as well as temporal and spatial distributions of expression to the donor plant, this is not guaranteed. For example, the length of the cis regulatory elements transferred as part of the cisgene to the recipient plant will likely impact on the expression pattern. Furthermore, in cisgenesis (as in conventional breeding and transgenesis) the expression of the introduced cisgene may for various reasons fall outside the range of expression variation observed in conventional plant varieties (Prins and Kok, 2010), if indeed the gene is already present in such varieties.

Intragenesis offer considerably more options for modifying gene expression and trait development than cisgenesis since genes and their promoters and regulatory elements are interchangeable within the intragenes. Therefore, the expression levels and patterns can be altered. Intragenesis can also include intragenes which target gene silencing, e.g. RNAi using within-species DNA sequences (Rommens, 2007; Rommens et al., 2007). The potential for gene silencing in plant breeding is not confined to transgenic, cisgenic and intragenic approaches, as alleles of silenced genes have been selected in conventional plant breeding (Manning et al., 2006; Martin et al., 2009; Weil and Martienssen, 2008).

4.5. Conclusions

Hazards that might result from various plant breeding techniques are related to the sources of genes used, the genes and traits deployed and changes to the structure, organisation and sequence of the genome which could have adverse consequences. The primary drivers are the genetic alterations that various breeding processes introduce into the plants, as all other changes that take place are direct or indirect consequences of these changes. Hazards regarding these elements may arise in conventional breeding, cisgenesis, intragenesis and transgenesis.

Cisgenesis and intragenesis do not introduce the range of other genes and sequences that can be associated with linkage drag in conventional breeding and thus avoid introducing the unwanted traits and hazards associated with these other genes/sequences. With respect to the introduced genes, the hazards arising from the use of a related plant-derived gene by cisgenesis are similar to those from conventional plant breeding, as similar traits are expressed by the gene. However when a related plant-derived gene is used in intragenesis some new combinations of genetic elements may arise that are not found in cisgenic and conventionally bred plants and these may present novel traits with novel hazards. Cisgenesis with T-DNA borders only differs from cisgenesis by the presence of short T-DNA border sequences. Similar sequences can be found in different plant species. Therefore, any hazards related to these sequences would not differ from those in conventional plant breeding.

Besides the hazards associated with the inserted genes and traits, this opinion also addresses the comparisons between cisgenesis, intragenesis, transgenesis and conventional breeding with regard to other changes to the genome caused by the processes by which the genetic elements are introduced.

Cisgenesis and intragenesis make use of the same transformation techniques as transgenesis. The potential for 'random' changes to the genome caused by the insertion event is, however, not limited to transgenesis, cisgenesis and intragenesis. Independent of breeding methodology, DNA is integrated into the plant genome by means of illegitimate recombination (via non-homologous end joining) into breaks. Mutational processes such as insertions/deletions/rearrangements of endogenous genes and regulatory sequences not only occur in transgenesis, cisgenesis and intragenesis but are phenomena that are known to occur in conventional breeding approaches. New open reading frames will be created at random during conventional breeding, cisgenesis, intragenesis and transgenesis and in all cases could give rise to new proteins. However, with transgenesis exogenous, non-host (and even non-plant) DNA is involved, possibly leading to the formation of sequence combinations and open reading frames which would normally not occur with conventional breeding or cisgenesis.

The regeneration of plants in tissue culture can be a major contributor to genetic and epigenetic changes (somaclonal variation), resulting in significant impacts on phenotype. This can occur during the development of cisgenic, intragenic and transgenic plants but also in conventional breeding and plant propagation whenever tissue culture processes are used. Undesirable phenotypes can be discarded by the breeder or may be eliminated by backcrossing where possible.

In summary, with respect to the trait the products developed using cisgenes are the same as those that could be produced using conventional breeding approaches. This is not necessarily the case for intragenesis and transgenesis. Cisgenes, intragenes and transgenes are introduced into the plant using the same transformation techniques and are integrated into the plant genome by mechanisms used by plants for natural DNA repair. In all of the cases, deletions and rearrangement and creation of new open reading frames can be expected as plant genomes are by their nature fluid entities. For intragenesis, new combinations of the DNA used could raise additional safety issues and make it more similar to the wider opportunities of transgenesis in this respect. Hazards can be identified which are specific for transgenic plants: (1) the transgenes and their gene products can be obtained from any source including non-plant; (2) there is the potential for producing new fusion proteins which would not be produced via conventional or cisgenic breeding due to the linking of non-host (including non-plant) sequences to the recipient plant's DNA.

While addressing question two of the mandate, the EFSA GMO Panel compared the hazards associated with plants produced by cisgenesis and intragenesis with those obtained either by conventional plant breeding techniques or by transgenesis. The Panel concludes that similar hazards can be associated with cisgenic and conventionally bred plants, while novel hazards can be associated with intragenic and transgenic plants (see sections 3, 4.1–4.5). The Panel found that all of these breeding methods can produce variable frequencies and severities of unintended effects. The frequency of unintended changes may differ between breeding techniques and their occurrence cannot be predicted and needs to be assessed case by case. Independent of the breeding method, undesirable phenotypes are generally removed during selection and testing programmes by breeders. The risks to human and animal health and the environment will depend on exposure factors such as the extent to which the plant is cultivated and consumed.

5. Applicability of the current guidance with respect to the risk assessment of cisgenic and intragenic plants and derived food/feed (addressing question one of the mandate)

The EFSA GMO Panel has detailed in its guidance documents how to comply with the requirements set out in Directive 2001/18/EC. In order to assess the adequacy of the current EFSA guidance documents for the risk assessment of cisgenic and intragenic plants the EFSA GMO Panel has focused on the *Guidance for risk assessment of food and feed from genetically modified plants* (EFSA, 2011) and the *Guidance on the environmental risk assessment of genetically modified plants* (EFSA, 2010). These guidance documents are based on the experience hitherto gained with transgenic plants. For GM plants the Panel reiterates that risk assessment is carried out on a case-by-case basis, which may require different amounts of data to be provided.

The EFSA GMO Panel is of the opinion that these two EFSA GMO Panel guidance documents for GM plants cover all of the elements and approaches that might be required to risk assess cisgenic/intragenic plants.

5.1. General background

5.1.1. History of safe use for consumption as food

The concept of “history of safe use” is an important concept in the comparative risk assessment for genetically modified plants (EFSA, 2011). In case a “history of safe consumption as food” is claimed, this should be specifically documented (Constable et al., 2007; Engel et al., 2011; Health Canada, 2006).

When determining whether or not “history of safe use for consumption as food” exists, it is important to verify whether experience and data available to address the various safety issues commonly assessed for GM plants are available (e.g. for components such as anti-nutrients, toxicants and allergens).

Particularly important in this regard is the exposure assessment, i.e. an estimation of the intake by man and/or animal of a component in question. It is also important to take into account the background level of intake so as to be able to judge whether, and by what magnitude, the change in component levels caused by cisgenesis/intragenesis/transgenesis will lead to an altered total intake of that component by the consumer.

5.1.2. Concept of familiarity

For environmental risk assessment, the underlying assumption of the comparative approach is that the GM plants are assessed using appropriate comparator plants with often well described biology. This led to the concept of familiarity, as developed by OECD (1993). In environmental risk assessment, it is appropriate to draw on previous knowledge and experience when using appropriate comparators in order to highlight the differences with the GM plant in its receiving environment(s).

5.1.3. Food and feed guidance for GM plants

The food and feed risk assessment of GM plants focuses on the intended and the unintended effects caused by the modification in order to elucidate whether they might pose a hazard to human and animals. The *Guidance for risk assessment of food and feed from genetically modified plants* (EFSA, 2011) is currently applied to genetically modified plants. Data from molecular characterisation and comparative analyses are used to check the potential occurrence of unintended changes of the genetic modification. The outcomes of the molecular characterisation can provide insight into potential sources of unintended effects, such as the disruption of endogenous genes resident at the location of the DNA insertion site.

5.2. Applicability of guidance on molecular characterisation to cisgenic and intragenic plants

The molecular characterisation section of the guidance for GM plants (2011) asks for the analysis of the insert and the flanking regions including the ORFs spanning the insert–plant genome junction. Considering that cisgenes are derived from the breeders’ gene pool and contain their own promoter and terminator, the rationale for some elements of the molecular characterisation should be reconsidered (e.g. ORF searches within the insert are not needed as no new internal junctions are present). Thus an update of the existing guidance on risk assessment should be considered to introduce additional flexibility. For example, with regard to the section of the molecular characterisation which deals with expression analysis, the EFSA GMO Panel acknowledges that some flexibility in the design of the trial might be needed.

5.3. Applicability of guidance on GM food feed assessment to cisgenic and intragenic plants

Hazards that might result from various plant breeding techniques are related to the sources of genes used, the genes and traits deployed and changes to the genome (see section 4), and could impact on human and animal health. Based on the origin of the cisgenes and the fact that the structure (DNA sequence) of the cisgenes in the recipient plant has remained unchanged compared to the donor plant, gene products similar to those in the donor can be expected in cisgenic/intragenic plants. Therefore, it can be envisaged that, for some elements of the food and feed safety assessment, there may already be sufficient information to complete a particular part of the risk assessment. In some cases the amount of new data to be generated to complete the risk assessment may be less extensive for cisgenic/intragenic plants when compared to transgenic products for which background information is often not available. With regard to the exposure assessment of cisgene-encoded proteins and associated metabolites, sufficient information may already exist on the quantities that are safely consumed. For example, in cases where it is well documented that both the donor plant and the newly expressed proteins in cisgenic/intragenic plants have a corresponding use and history of safe consumption as food and feed, specific toxicity testing may not be required if the intake levels are within a range considered to be safe. However, if the intake levels are outside of this range, further safety assessment is needed.

The safety of both intended and unintended changes due to the genetic modification is assessed for GM plants and derived food and feed. For plants derived through cisgenesis/intragenesis, the EFSA GMO Panel considers that the general approach and all elements described in the guidance for risk assessment of food and feed from GM plants (EFSA, 2011) is, at the present time, sufficient for the evaluation of cisgenic/intragenic plants and derived food and feed. However, for the assessment of food and feed products derived from cisgenic plants and intragenic plants it can be envisaged that, on a case-by-case basis, lesser amounts of event-specific data are needed. For example relevant information might already be available regarding the nature of the cisgenic/intragenic traits and/or plant products, experience with the donor and/or recipient plants and the history of safe use and/or consumption (see section 5.1).

5.4. Applicability of guidance on GM environmental risk assessment to cisgenic and intragenic plants

The EFSA GMO Panel considers that all elements described in its guidance on the environmental risk assessment of GM plants (EFSA, 2010) are relevant for, and can apply to, cisgenic and intragenic plants. The relevance of applying specific elements of the guidance on the environmental risk assessment to cisgenic/intragenic plants is determined on a case-by-case basis. Based on the characterisation of the plant, traits, receiving environments, its intended uses, changes in management, and combinations of these factors, applicants shall apply those elements of the guidance that are relevant to the cisgenic/intragenic plant under consideration. Problem formulation, including hazard identification, as the critical first step of the environmental risk assessment, drives the selection of relevant specific elements and hence the requirements for the environmental safety assessment for each cisgenic/intragenic plant. However most “trait-related” areas of concern, such as the

consequences of changes in management, arising from each of these techniques will have similar data requirements. Problem formulation allows a structured, logical approach to identifying the important environmental issues meriting detailed risk characterisation.

Risk assessment data requirements for cisgenic/intragenic plants can be reduced on a case-by-case basis where there is familiarity with the donor and/or recipient plants, as well as cis-/intra-genes and their products. This familiarity and existing knowledge help to reduce the level of scientific uncertainty associated with the novel plant and hence the number of hypotheses to be tested in the risk characterisation phase.

Unintended effects on the environment of cisgenic/intragenic plants can be considered as for GM plants. Thus the weight of evidence approach described in EFSA (2010) can be followed and hence information on the molecular characterisation, compositional analysis, agronomic and phenotypic characterisation and *in planta* data on plant–environment interactions can be examined (EFSA, 2010) and compared with appropriate comparators. Any statistically significant differences found between the cisgenic/intragenic plant and its comparators can be assessed specifically for their biological relevance and environmental impacts.

The EFSA GMO Panel confirms that all elements described in its guidance on the environmental risk assessment of GM plants can apply to cisgenic/intragenic plants, and the relevance of applying specific elements of the guidance is defined on a case-by-case basis.

5.5. Conclusion

The EFSA GMO Panel considers that the general approach and all elements described in the *Guidance for risk assessment of food and feed from genetically modified plants* (EFSA, 2011) and the *Guidance on the environmental risk assessment of genetically modified plants* (EFSA, 2010) are applicable for the evaluation of food and feed products derived from cisgenic and intragenic plants and for performing an environmental risk assessment and do not need to be developed further. It can be envisaged that on a case-by-case basis lesser amounts of event-specific data are needed for the risk assessment.

CONCLUSIONS

The European Commission asked the EFSA Panel on Genetically Modified Organisms to deliver a scientific opinion on plants developed through cisgenesis and intragenesis in terms of the risks they might pose and the applicability of the existing guidance documents for their risk assessment. The mandate included two specific questions:

1. Determine whether there is a need for new guidance or whether the existing guidance on risk assessment should to be updated or further elaborated, in anticipation of the placing of products on the market through the application of the listed techniques.
2. What are the risks in terms of impact on humans, animals and the environment that the eight techniques listed could pose, irrespective of whether or not they fall under the GMO legislation? This latter request should consider the most recent scientific literature and knowledge of plant breeding experts and compare plants obtained by these new techniques with plants obtained by conventional plant breeding techniques and secondly with plants obtained with currently used genetic modification techniques.

The EFSA GMO Panel considers that the *Guidance for risk assessment of food and feed from genetically modified plants* (EFSA, 2011) and the *Guidance on the environmental risk assessment of genetically modified plants* (EFSA, 2010) are applicable for the evaluation of food and feed products derived from cisgenic and intragenic plants and for performing an environmental risk assessment and do not need to be developed further. It can be envisaged that on a case-by-case basis less event-specific data are needed for the risk assessment.

While addressing question two of the mandate, the EFSA GMO Panel compared the hazards associated with plants produced by cisgenesis and intragenesis with those obtained by either conventional plant breeding techniques or by transgenesis. The Panel concludes that similar hazards can be associated with cisgenic and conventionally bred plants, while novel hazards can be associated with intragenic and transgenic plants. The Panel is of the opinion that all of these breeding methods can produce variable frequencies and severities of unintended effects. The frequency of unintended changes may differ between breeding techniques and their occurrence cannot be predicted and needs to be assessed case by case. Independent of the breeding method, undesirable phenotypes are generally removed during selection and testing programmes by breeders. The risks to human and animal health and the environment will depend on exposure factors such as the extent to which the plant is cultivated and consumed.

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GLOSSARY

<i>Agrobacterium tumefaciens</i>	A naturally occurring pathogenic bacterium of plants that can incorporate a part of its DNA into plant cells.
Allele	One of two or more forms of a gene occupying the same locus on a particular chromosome or linkage structure and differing from other alleles of that locus at one or more mutational sites (Rieger et al., 1968).
Breeders' gene pool	The sources of genes available for conventional plant breeding.
Callus	An undifferentiated mass of cells that initially arises from plant cell or tissue in artificial culture.
Chromatin	A substance with characteristic staining properties distributed throughout the interphase nucleus. Chromatin is that part of the nuclear material that makes up the genetic material and contains the genetic information of the cell (Rieger et al., 1968).
Cisgenesis	Cisgenesis is the genetic modification of a recipient organism with a gene from a crossable - sexually compatible – organism (same species or closely related species). This gene includes its introns and is flanked by its native promoter and terminator in the normal sense orientation.
Cisgenic plant	Plant created using technique of cisgenesis.
Cisgene	Gene introduced into a recipient organism by genetic modification and originating from a crossable - sexually compatible – organism (same species or closely related species). This gene includes its introns and is flanked by its native promoter and terminator in the normal sense.
Conventional plant breeding	Within the context of this document conventional plant breeding is defined as methods used by plant breeders for the improvement of commercial varieties and where the resulting plants/varieties are not covered by Directive 2001/18/EC. The term is used interchangeably with traditional plant breeding (see Van der Wiel et al. 2010).
Cross-breeding	Mating between members of different populations (lines, breeds, races, or species).
DNA	Deoxyribonucleic acid is a polymer of deoxyribonucleotides which is the primary genetic material (Rieger et al., 1968).
Endogenous	Originating within the organism, cell, or system being studied (Lawrence, 1995).
Epigenetic	Refers to alterations of gene activity without altering the nucleotide sequence or genotype of an organism.
Gamete	A mature reproductive cell capable of fusing with a cell of similar origin but of opposite sex to give a zygote (Rieger et al., 1968).
Gene	A segment of nucleic acid that codes for a protein or RNA with a defined function in the structure or metabolism of the organism, and containing the regulatory sequence elements for its expression. The unit of inheritance (Glick and Pasternak, 2003).
Genetically modified	Refers to an organism whose genotype has been altered in a way that does not naturally occur (see Directive 2001/18/EC).

Genome	The entire complement of genetic material of an organism, virus, or organelle; in eukaryotes, the haploid set of chromosomes (adjusted from Glick and Pasternak, 2003).
Hazard	Biological, chemical and physical agents capable of causing adverse health effects and which may be present in a particular food and feed or group of foods and feeds.
Homologous recombination	Recombination between similar DNA sequences (Lawrence, 1995).
Hybrid	Any offspring of a hybridisation or cross between two genetically unlike individuals (Rieger et al., 1968).
Inbred line	Any more or less homozygous line derived from an outbreeding population by repeated inbreeding of an individual and its progeny (Rieger et al., 1968).
Intended effects	Intended effects are those that fulfil the original objectives of the modification.
Interspecific incompatibility	Any pre-zygotic (e.g. inhibition of pollen germination) or post-zygotic (e.g. embryo abortion) process preventing the formation of hybrids.
Intragenesis	Intragenesis is a genetic modification of a recipient organism that leads to a combination of different gene fragments from donor organism(s) of the same or a sexually compatible species as the recipient. These may be arranged in a sense or antisense orientation compared to their orientation in the donor organism. Intragenesis involves the insertion of a reorganised, full or partial coding region of a gene frequently combined with another promoter and/or terminator from a gene of the same species or a crossable species.
Intragenic plants	Plant created using technique of intragenesis.
Introgene	A gene construct that contains all elements from donor organism(s) of the same or a sexually compatible species as the recipient.
Landrace	A primitive cultivar (in contrast to a named modern cultivar). Landraces of a particular plant are a collection of plants that were developed and maintained by traditional farmers. While they are genetically improved over wild versions of the species, they are not as genetically fixed and homogeneous as modern commercial cultivars.
Linkage drag	Co-transfer of DNA sequences that are linked to the gene of interest.
Locus	The position of a gene on the genetic map (Rieger et al., 1968).
Meiosis	The special cell division process by which the chromosome number of a reproductive cell becomes reduced to half (n) the diploid (2n) or somatic number.
Methylation	Addition of a methyl group (-CH ₃) to a macromolecule, such as a specific cytosine and, occasionally, adenine residues in DNA.
Mitochondrion (pl.:mitochondria)	The eukaryotic organelle, with endosymbiotic origin, involved in aerobic respiration, particularly the citric acid cycle, respiratory electron transport and oxidative phosphorylation (adapted from Mauseth, 1991).
Mutations	Any detectable and heritable change in the genetic material not caused by segregation or genetic recombination, which is transmitted to daughter cells and even to succeeding generations giving rise to mutant

	cells or mutant individuals provided it does not act as a dominant lethal factor (Rieger et al., 1968).
Organelle	Any structure of characteristic morphology and function within the cytoplasm of the cell (Rieger et al., 1968).
ORF	Any nucleotide sequence that consists of a string of codons that is uninterrupted by the presence of a stop codon in the same reading frame.
Pan genome	The pan genome concept defines core genomic features that are common to all individuals in the species and a dispensable genome composed of partially shared and/or non-shared DNA sequence elements.
Phenotype/Phenotypic	The observable properties (structural and functional) of an organism, produced by the interaction between the organism's genetic potential (genotype) and the environment in which it finds itself (Rieger et al., 1968).
Plastid	A cytoplasmic organelle in plant cells, primarily involved in the formation (via photosynthesis) and storage of soluble and insoluble carbohydrates (Rieger et al., 1968).
Polyploid	Somatic cells and tissues, as well as individuals, having three (triploid), four (tetraploid), five (pentaploid), or more complete chromosome sets instead of two as in diploids (Rieger et al., 1968).
Problem formulation	Each risk assessment (EFSA, 2010) begins with problem formulation in which the most important questions that merit detailed risk characterisation are identified. Problem formulation helps to make the risk assessment process transparent by explicitly stating the assumptions underlying the risk assessment.
Promoter	A segment of DNA to which RNA polymerase attaches, allowing the initiation of the transcription of a gene. It usually lies upstream of (5' to) a gene (adjusted from Glick and Pasternak, 2003).
Protoplasm	All the substance of a cell, excluding the cell wall (adapted from Mauseth, 1991).
Protoplast	The protoplasm of a single cell, obtained by the enzymatic digestion of the cell wall (adapted from Mauseth, 1991).
<i>R</i> gene	Resistance gene.
Recombination	Any process which gives rise to cells or individuals associating in new ways two or more heritable determinants by which their parents differed (Rieger et al., 1968).
Risk characterisation	Risk characterisation is defined as the qualitative and/or quantitative estimation, including attendant uncertainties, of the probability of occurrence and severity of known or potential adverse health effects in a given population based on hazard identification, hazard characterisation and exposure assessment.
Segregation	The separation of allele pairs from one another and their distribution to different cells, usually at meiosis and sometimes at mitosis (Rieger et al., 1968).
Silencing	Shutdown of transcription of a gene.

Somaclonal variation	Genetic variation arising from mutations, or heritable epigenetic variation, in somatic plant cells undergoing regeneration in culture.
Somatic tissue	Tissues other than the germline.
T-DNA	DNA encoded on a plasmid of <i>Agrobacterium</i> that is transferred to the plant cell.
Trait	A phenotypic character of an organism.
Transgenesis	The use of recombinant DNA techniques for the introduction of genetic information into animal or plant cells that leads to the transmission of the input gene (transgene) to successive generations.
Transgenic plant	Plant developed using by transgenesis.
Transgene	A gene from one source that has been incorporated into the genome of another organism by transgenesis.
Transposon	A DNA element capable of moving (transposing) from one location in a genome to another in the same cell through the action of transposase.
Unintended effects	Unintended effects are consistent differences between the plant under consideration and its comparator, which go beyond the intended effect(s) of the modification.
Variety	Is used here interchangeably with cultivar.