CO-EXTRA

GM and non-GM supply chains: their CO-EXistence and TRAceability

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1 Summary

This deliverable is a position document (report) in which the authors have reviewed and discuss the definition of gene stacking in plants, and its implications in relation to genetics, safety, detectability and European regulations. The purpose is to provide the stakeholders with a better fundament for establishing more coherent definitions and requirements and to recognize and appreciate the limitations, advantages and drawbacks linked with different ways of conceptualizing gene stacking. The work has been performed in workpackage 6 (WP6) of the Co-Extra project.

In the Description of Work (DoW) of the Co-Extra project, the nature of this deliverable was specified as a report that shall be publicly available. However, as the authors wish to publish this report in a slightly revised form as a peer-review publication, a manuscript was submitted within one month after submission of the present report. The authors have requested the project managers to accept that the present report is confidential and restricted to project partners and the European Commission, and that the peer review publication will act as the official publicly available report.

The report has primarily been prepared by researchers from the Instituut voor Landbouw- en Visserijonderzoek (ILVO), Merelbeke, Belgium (partner 22), with some contribution also from the National Veterinary Institute (NVI), Oslo, Norway (partner 7), and a minor contribution from the Institute National de la Recherche Agronomique (INRA), Versailles, France (partner 1).

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2 Introduction

The cultivation and breeding of biotech crops accelerated in the beginning of the second decade of their adoption. In 2006 the global area of approved biotech crops reached 102 million hectares up from 90 million hectares in 2005. The principal biotech crop remains soybean (52% of the global biotech area), followed by maize (25% of the global biotech area), cotton (13% of the global biotech area) and canola (5% of the global biotech area). The dominant trait during the first decade was herbicide tolerance, followed by Bt-toxin induced resistance to insects and products combining both types of feature. Such combinations are often referred to as “stacking” or “pyramiding” of traits, although recently other terms have also been used. The cultivation of “stacked traits” or more precisely “gene stacked GMOs”, also called “stacked GMOs”, “stacked events” or shortly “stacks”, is expected to increase in near term, with an introduction of new traits to meet the needs of the consumers and producers (James 2006).

The production of stacked events aims at combining different new agronomic traits by introducing different novel genes in the plant genome. Stacked traits are produced in many organisms via different strategies. As is the case for any transgenic event, different steps precede the final authorization and commercialization of a stacked transgenic event. Selection of elite events is followed by commercial seed production and propagation. Upon authorization and market introduction by cultivar registration, seed and/or grain processing will lead to food and feed products which contain, or are partly or completely derived from stacked transgenic events. In addition to intended (“commercial”) gene stacks (cGS) resulting from a desire to produce stacked seeds, unintended gene stacks can develop when two different GMOs hybridize in the field, resulting in “wild” gene stacks (wGS).

Related to the different ways taxa are transformed and stacked events produced, including classical breeding, and to differences in global authorization and commercialization practices, different types and definitions of gene stacked events exist. Seen from a number of different contexts, different common perceptions of stacking exist. This paper aims at making the terminology regarding stacked events more comprehensive, in trying to limit and group terms in relation to various implications and consequences of the definitions.

Within the EU, transgenic lines containing stacked traits have to be approved according to the EU GMO legislation which comprises a step-by-step approval process. The basis for approval is a case-by-case risk assessment with regard to the human health and environment (EC, 2001; 2003a), availability of methods for detection and reference materials (EC, 2004a), traceability (EC, 2003a; 2003b), and post-market monitoring (EC, 2001). Outside the EU, other rules and practices with regard to the authorization of stacked events may exist. A part of this report is a summary review of non-EU regulations in this context.

Regulatory and analytical issues may differ globally, with respect to how cGS and wGS are tackled. This may have a strong impact also on the possibility to define unique, if possible, genetic markers for gene stacked GMOs. This review is meant to provide a basis to determine if such genetic markers can be defined, what their nature may eventually be, and how they may be made detectable and possibly quantifiable. Notably, a core element in the EU-legislation is provision of a validated event-specific quantitative detection method by the notifier prior to authorisation for placing on the market of any GM feed or food. This method serves partly to facilitate labeling, but also as a tool to monitor potential unintended dispersal of the GMO.

The overall objective of this position document is to review the challenges, limitations and potential solutions relative to gene stacking. Given the nature of stacked gene events, stakeholder face some serious challenges linked with current legislation, in order to detect, identify and quantify stacked gene events in plant products. Here we describe the methods of production of stacked gene traits, the status of their cultivation and approval, and methods for detection and quantification. We discuss the problems related to their detection and quantification as it is required from the current EU legislation, and the available and foreseeable solutions.

This task is related to another Co-Extra task T4.4 (Survey, analysis and development of cost-effective and fit-for-purpose sampling plans and guidelines), and more specifically in that context to trying to detect stacked genes by a statistical (sub-sampling) strategy by qualitative and quantitative PCR.

3 Gene stacked GMOs - Definitions and terminology

3.1 General definitions reflecting the most common perception of gene stacks

The most common and general perception of gene stacking is that, if more than one gene of interest from another organism has been transferred, then the created GMO has stacked genes (or stacked traits). Gene stacking has become important in plant breeding. Occasionally, researchers wish to transfer more than one trait of interest (e.g. an insect resistance and a herbicide tolerance) to a crop. Consequently, they need to transfer more than one gene, and do so either in one or in subsequent steps. This can beindeed achieved either by genetic engineering or by conventional cross-breeding of GM plants with two different modifications.

According to the Organisation for Economic Co-operation and Development (OECD), stacked transformation events are defined as “new products with more than one transformation event” (OECD, 2004). This is also the definition which is widely accepted by breeders. This official definition is both very broad and yet may be too limited, as will be discussed in more detail later, and it clearly implies that the term “transformation event” needs to be well defined (see also Lezaun, 2006).

Gene stacking or pyramiding is a strategy applied in agriculture to improve the agronomic properties of the crop, e.g. achieving multiple virus resistance by pyramiding virus resistance genes in barley varieties (Werner *et al.*, 2005). The stacking of GM traits provides advantages for the producers to obtain expressions of multiple transgenes, enhancing the different uses of the plant.

In summary, the most common and basic perception of gene stacking is that it is used to produce GM events containing and expressing different transgenic traits and thereby combining/enhancing different properties of the plant (e.g. multiple resistances). This perception primarily refers to the phenotype of the plant: “stacking” refers to the natural (i.e. by genetic crossing) addition of different plant properties previously obtained by different independent taxon transformations. With different is meant: more than one transformation event, each of which is either coming from a different source (e.g. donor organism) and/or conferring different properties (e.g. tolerance to a herbicide and resistance to insects). As it will become evident when looking at the level of the genotype in the following, a consistent scientific definition of gene stacking and a gene stacked event is much more complicated. Some of the most frequently and widely used terms related to stacking are listed in **Table 1**.

Table 1. Summary of terminology used for gene stacks, relative to the process of production and to the resulting event (from Halpin, 2005).

Process of production	**Resulting event**
Stacking	gene stack (GS), stack, stacked trait, stacked event, gene stacked event (GSEv)
Pyramiding	pyramided event (equivalent to stacking) obtained by natural crossing of two transformation events
Co-transforming	multitrait or combined-trait event with one single insert
Multiple or re-transforming	multitrait or combined-trait event with separate inserts
Multigene introduction	co- or multiple transformants obtained from concomitant or successive insert introductions

3.2 Definitions according to the transformation/production strategy used

Halpin (2005) defined "effect" genes as genes intended to add some useful change in the final plant product, as opposed to selectable marker genes which may only be used for the initial selection of transformed material. The terms "gene stacking" and "stacked traits" primarily refer to the phenotypic characteristics of the transgenic plant, namely introducing two or more effect genes coding for different traits (see above). However, different strategies can be applied to produce events with stacked genes, giving rise to different patterns at the genotype level of the plant.

When considering the different approaches for gene stack production, it is useful to elaborate on the terminology concerning 'transformation events', proposed by Holst-Jensen et al. (2006). According to these authors, the most widely used and basic term to refer to a specific GMO, is probably "transformation event" (TraEv). "Transformation", i.e. the process of modification of a cell by the uptake and incorporation of exogenous (foreign) DNA, leads to the establishment of one or more "modified sequence(s)" (ModSeq) in the modified cell. The ModSeq is the result at molecular level, i.e. insertion of new/modified genetic information in the genome of the modified cell (organism). This definition was limited to a single functional genetic construct, also covering vector elements and non-expressed genes (e.g. selectable marker genes with bacterial promoters inactive in a plant) possible rearrangements, insertions/deletions, or substitutions of a part of the genome of the cell. A "gene construct" (construct) may be defined as a combination of genetic elements serving the purpose of activation and regulation of the expression of the gene which is part of the construct before and after integration in the genome of the recipient organism. This definition implies that vector elements and non-expressed genes (e.g. selectable marker genes with bacterial promoters inactive in a plant) are not considered as parts of constructs.

An alternative definition to ModSeq could be "integrated sequence" (InSeq) that could include non-functional/non-expressed inserts, such as partial/truncated constructs. It should be noticed that Halpin's (Halpin, 2005) definition of "effect" genes has some limitations. In many cases the expression of the selectable marker introduces a change in the final plant product although the intended effect is different (e.g. maize events carrying insect resistance and a herbicide tolerance as a selectable marker, **Table 2**, Annex 1).

Following the terminology of Holst-Jensen et al. (2006), the results of transformation at plant level are "transformation events" (TraEv), defined as the primary transformants, which are always heterozygous for the ModSeq. As TraEvs, a distinction was made between "unique events" (UniEv) or single events, and "multiple events" (MulEv). Unique events carry a single-copy single functional ModSeq, or a ModSeq at one locus. Multiple events carry multiple-copy and/or multiple functional ModSeq, at one locus or at different loci. Where the term TraEv is used for the primary transformants only (referring to the transformation process as such), the authors refer to "stacked events" (StaEv) as secondary transformants, obtained after crossing single TraEvs. Holst-Jensen et al. (2006) thus distinguish between a StaEv and a MulEv. Stacking is used *sensu stricto* and refers to the conventional "crossing" of different transformation events, i.e. the result of hybridization between two previously independent events, where the progeny carries at least one ModSeq from each parent event. According to Holst-Jensen et al. (2006), gene stacking leads to the creation of an offspring containing the inserted genetic constructs of both parental TraEvs, whereby the derived plant contains two or more physically unlinked (with independent Mendelian segregation) inserted genetic constructs. They remarked, however, that from the analytical detection – and thus molecular or genotypical – global point of view, there is no difference between these StaEvs as a result of crossing, and MulEvs obtained by transformation with two or more transformation vectors.

The TraEvs (primary transformants) undergo several further steps before to the developer applies for authorization and marketing of the newly produced transgenic event. TraEvs pass several cycles of backcrossing with elite inbred line and several cycles of self pollination to obtain homozygous transgenic elite events (EliEvs). The elite events can subsequently be crossed to obtain gene stacks (StaEvs). The F1 hybrid transgenic seeds may then undergo different backcrosses with the parental elite inbred seed line, leading to introgression of the desired (GM) trait in the elite background. The stacked F1 hybrid transgenic seed may be used as commerce seed (ComSe; hemizygous for the inserted transgene). Selfing of this F1 seed then results in F2 GM seeds as ComSe (usually

hemizygous for the trait). These F2 seeds are commercialized and enter the market, being used further in cultivation and processing to food and/or feed derived products.

For the production of gene stacks, Halpin (2005) basically distinguishes multiple introductions of single transgene constructs from single introduction of multiple transgenes. Iterative strategies refer to the sequential/multiple introduction of 'single transgene constructs' into one plant. This can be achieved by (a) crossing single plants containing single transgenes (i.e. the OECD definition of stacked genes), or (b) re-transformation(s) of a single transgene plant with additional transgenes. Examples of stacked transgene events produced by crossing are current maize and cotton events conferring genes for herbicide tolerance and insect resistance with a current introduction of up to 4 transgenes in a final breeders' seed (a ComSe). Examples of stacked gene events produced by re-transformation are cotton event 15985 with enhanced insect resistance and potato events conferring genes coding for both insect and virus resistance (**Table 2** in Annex 1).

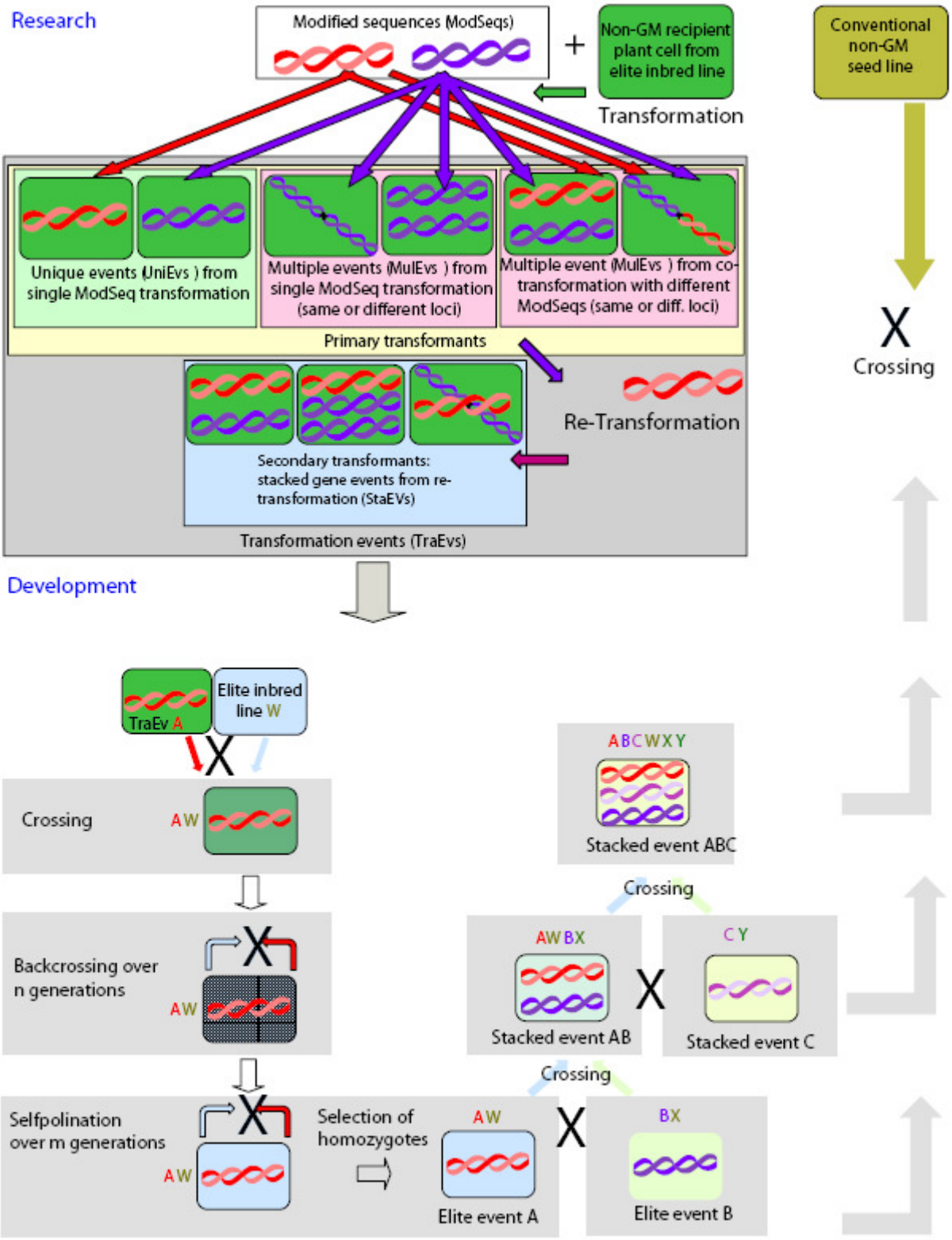
Co-transformation methods are used for the simultaneous introduction of 'multiple constructs' into one plant: either by (a) transformation with a plasmid construct carrying several genes of interest, a growing practice in biotech companies, i.e. assembling multigene cassettes and introducing these multiple constructs on different T-DNAs, or by (b) co-transformation with different plasmids carrying different transgenes, or multiple DNA fragments introduced to the plant cell either via *Agrobacterium tumefaciens* mediated transformation or biolistic methods. By this strategy the transgenes may insert in a single locus, e.g. genomic hot spots of recombination, but there are also cases with insertions into multiple loci (e.g. maize event Mon832).

Examples of stacked transgene constructs which are produced by co-transformation are maize "stacks" containing the traits herbicide tolerance and insect resistance or multiple insect resistances (see **Table 2** in Annex 1) and some non-commercialized rice stacks conferring multiple insect resistance (Cummins 2004). This may yield very complicated insertion patterns as observed for maize Bt176 (Novartis, 1997).

For natural, conventional, crossing-derived gene stacking (OECD definition of stacked genes), it may, as mentioned earlier, be relevant to discriminate between intended (commercial) gene stacks (cGS) and unintended hybridization that may take place in the field, resulting in a "wild" gene stack (wGS).

From the viewpoint of classical breeding, i.e. when considering stacks that are produced by conventional crosses, a "GM stacked event" is distinguished from a "GM hybrid" (De Schrijver et al., 2007). In a GM hybrid, the transgenic trait originates from a GM inbred parental line that was crossed with one (or more) non-transgenic elite inbred line(s). In a GM stacked event, two or more transgenic traits are brought together by crossing GM in-bred lines each being different initial events. For the latter, De Schrijver et al. (2007) define "one-way GM stacked events" as stacked events where two transgenic traits are combined, while 'three-way GM stacked events' contain three transgenic traits. The current situation in the USA is an increasing proportion of "four-way stacked events".

The different ways to produce GS, as described above, are summarized in **Figure 1.**


Research
Modified sequences (ModSeqs)
+
Non-GM recipient plant cell from elite inbred line
Transformation
Conventional non-GM seed line
Unique events (UniEvs) from single ModSeq transformation
Multiple events (MulEvs) from single ModSeq transformation (same or different loci)
Multiple event (MulEvs) from co-transformation with different ModSeqs (same or diff. loci)
Primary transformants
X
Crossing
Re-Transformation
Secondary transformants: stacked gene events from re-transformation (StaEVs)
Transformation events (TraEvs)
Development
TraEv A
Elite inbred line W
Crossing
A W
ABCWXY
Stacked event ABC
Crossing
Backcrossing over n generations
A W
A W B X
Stacked event AB
X
C Y
Stacked event C
Crossing
Selfpolination over m generations
A W
Selection of homozygotes
A W
Elite event A
X
B X
Elite event B

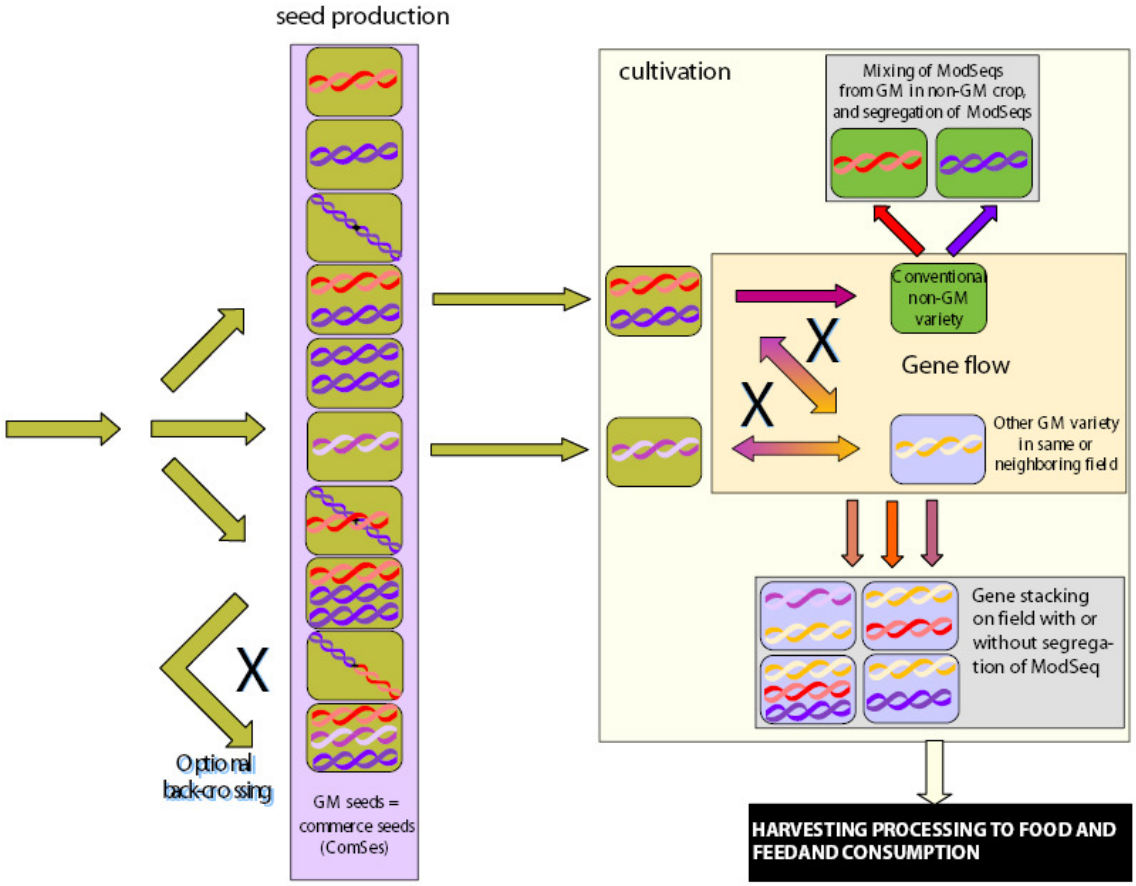


Figure 1. Previous page and above. Diversity of genetic origins and structures of constructs (TraEvs). The figure illustrates the basic principles of transgenic plant cell line development through all stages from the primary transformation (**top, previous page**) to gene flow, hybridization and gene segregation in the field during cultivation (**above**, **right**). Elements in grey boxes all represent various types of TraEvs, the term most often referred to as the object of governmental regulations (Lezaun, 2006). **(1) Research stage (previous page, above)**: A ModSeq can be inserted by transformation and integrate in a single copy or in multiple copies (complete or not, rearranged or not) in one or more loci in the genome. Integration of multiple copies of a ModSeq will result in a MulEv. Depending on the definition of a ModSeq (sequence contig or single effect-gene construct) a ModSeq may or may not confer more than one gene, and consequently insertion of a single ModSeq may or may not result in a multi-trait phenotype. By *co-transformation* two different ModSeqs are simultaneously inserted and tend to integrate in a single locus in the genome, although multi-loci insertion is also possible. *Re-transformation* of a single ModSeq line results in the integration of another ModSeq in a different locus. This process will create multi-trait phenotypes, that may be defined as gene stacked (GS), i.e. stacked events (StaEvs). **(2) Development stage (previous page, bottom)**: TraEvs undergo several cycles of backcrossing and self pollination to obtain homozygous lines from which the line with optimum performance of the trait is selected. These events called EliEvs are the units subject to authorization, risk assessment, quality assurance and quality control. The elite events can be crossed in order to obtain double or triple or multiple GS (StaEvs).**(3) Post-marketing stage (this page)**: The obtained TraEvs (including StaEvs) are crossed with a conventional (non-GM) seed line to produce commercial seeds (**left**), further used for cultivation and production of products for food and feed purposes. As a result of cultivation (**right**), non-intended gene stacking can occur due to gene flow from one event to another transgenic plant (wGS). This cycle of non-intended gene stacking can be repeated and plants containing more than two stacked traits can accidentally be obtained. Modified from Holst-Jensen et al. (2006).

From the phenotypic point of view, “stacking” refers to the presence of different transgenic traits, and thus “multiple events” or multiple ModSeqs. Similarly, the OECD definition refers to the presence of different “transformation events”. Given this broader definition, the term stacking refers to any process or situation where more than one ModSeq is present in one plant genome, albeit as a result of direct transformation (first level), or of conventional crossing of first-level transformation events (second level).

3.3 Definitions related to risk assessment and detection/identification of GS

The above mentioned distinction between ‘GM hybrid’ and ‘GM stacked event’ is important in light of risk assessment of new GMOs. The EFSA opinion on the risk assessment of plants containing genetic modifications combined by crossing, considers “stacks” as F1 hybrids between two elite lines each containing a single transgenic event, where in general meaning, the term “hybrid” covers the F1 generation of two genetically different plants, lines, cultivars, species or genera, including two different transgenic lines (EFSA 2006b). The risk assessment of stacked gene events in case of existing evaluation of the parental lines, is focused on the following items: assessment of the intactness of the integrated loci, phenotypic stability and expression of the events, and assessment of the potential interactions between the combined events.

Following the terminology proposed by Holst-Jensen et al. (2006), the term “transformation event” (TraEv) is used for any event that is the direct result of transformation, while “stacked event” (StaEv) is used for a further-level multiple event obtained by crossing two parental transformation events. Given the different strategies by which stacked events can be produced (see 3.2 and **Fig. 1**), and when applying this distinction, StaEvs can also be TraEvs (see **Fig. 2**). This means that from the molecular/genotypical point of view, the term “stacked event” refers to all events where multiple ModSeqs are present in the plant genome.

From the analytical point of view, the number and the structure of the integration loci are crucial. Assays to detect the transgenic event(s) are designed based on the molecular structure of the transgenic locus. Here the distinction to be made is between (1) two or more transgenes being introduced in a single locus and (2) multiple transgenes integrated in multiple loci in the plant genome.

Introduction of two or more genes will result in different integration patterns depending on the approach used. The introduction of more than one transgene in the plant genome can result in one or more transgene loci, depending on the method used to generate the GS (**Fig. 2)**.

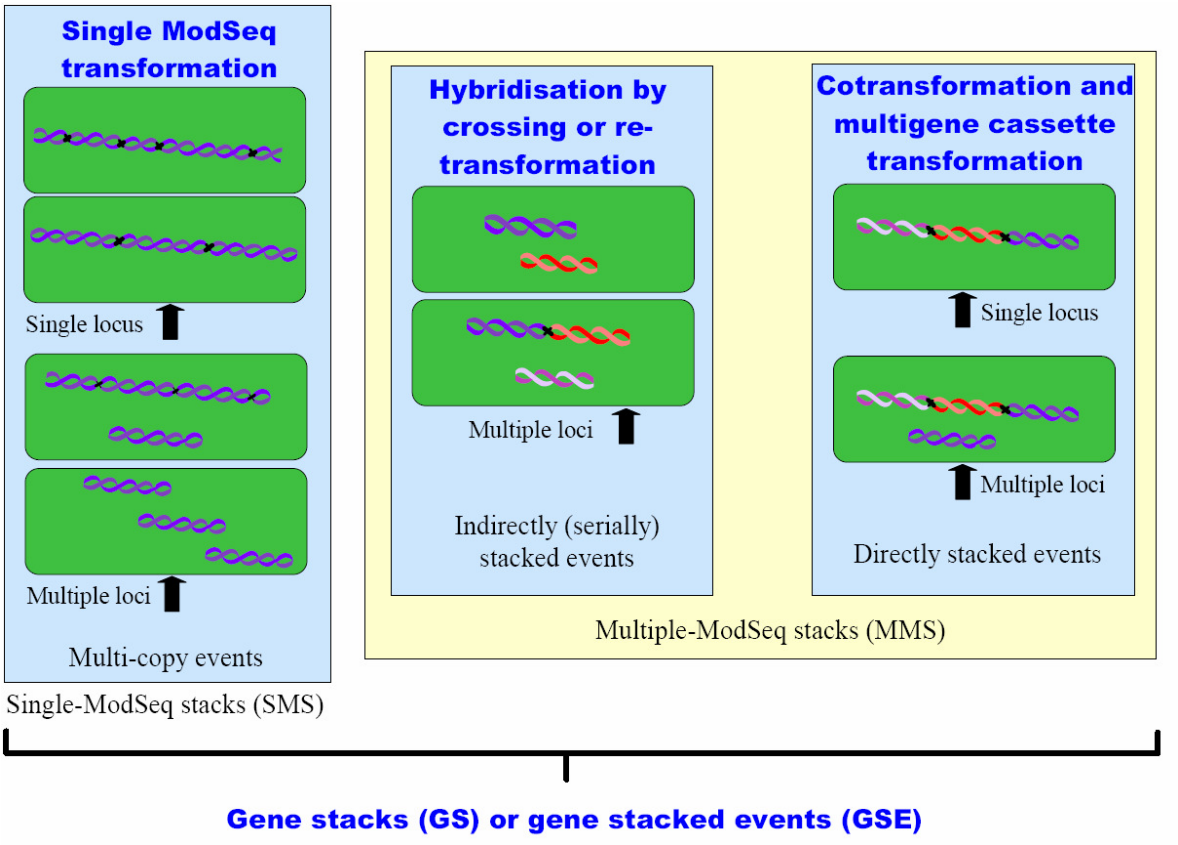


Figure 2. Terminology for gene stacking, seen from the analytical detection viewpoint: molecular structure of the integration locus/loci, resulting from different ways to produce GS. Co-transformation and transformation with multiple gene cassettes (directly stacked events) often lead to integration of different transgenes into a single locus in the host genome (**right part**). Indirectly stacked events, produced by cross hybridization and re-transformation, will contain different transgenes that are physically unlinked (**middle part**). At the molecular level neither of the two categories are considered as a single-copy event and more than one transformation event-specific PCR assay will be necessary to detect the different transgenes or transgene events. Both types of StaEvs, in the meaning of "multiple-ModSeq stacks" (MMS), need to be distinguished from a third category where genes are stacked: the "single-ModSeq stacks" (SMS, **left part**). A single transformation process (e.g. co-transformation) can lead to multiple dispersed and/or partial insertions of (parts of) the same gene. Multiple T-DNA copies can be introduced as a result of the transformation of a single transgene. Seen from the analytical level, both SMS (**left**) and MMS (**middle** and **right**) contain multiple genes or parts of genes, and therefore can be categorized under gene stacks (GS) or gene stacked events (GSE).

Iterative or multiple transformation strategies give rise to introduced transgenes which are not linked, but situated at different, random loci in the plant's genome. As a consequence, the introduced transgenes can segregate in subsequent generations. Co-transformation or single transformation methods generally result in transgenes that tend to co-integrate at the same chromosomal position and therefore will be inherited together in most progeny (Halpin, 2005).

In co-transformation, along with the presumed major insertion locus containing the different transgenes assembled in the (multigene) cassette used for transformation, also multiple dispersed insertions have been reported, e.g. in the case of Bt176 maize. According to Holst-Jensen et al. (2006), the latter would be defined as a "multiple event" (MulEv), but not as a "stacked event" (StaEv). The OECD guidelines for designation of unique identifiers for transgenic plants (OECD, 2004) are inconsistent at this point, although this inconsistency is discussed (Item 8 in the guide). For example, the MulEv Bt176 maize is identified by the OECD code SYN-EV176-9 (176), while the StaEv T25 x

Mon810 is identified by the OECD code ACS-ZMØØ3-2 x MON-ØØ81Ø-6 (see also **Table 2**, Annex I). Effectively, this means that while progeny of the StaEv can be accurately described by reference to their parental OECD codes if the transgenes segregate, the same is not the case with the MulEv. As a consequence the same code may apply to substantially different transgenes. This was probably not intended when the concept of unique identifiers was developed.

The terminology proposed by Holst-Jensen et al. (2006) does not distinguish the events produced by re-transformation and containing more than one transgene in more than one insertion locus. The same inconsistency occurs in the OECD definitions. For example the cotton event 15985 (OECD identifier MON-15985-7) contains three transgenic inserts integrated in three different sites in the genome: 1 complete and one incomplete copy of the *cry1Ac* insert and one copy of *cry2Ab* (AgBIOS data base, see **Table 2**, Annex I).

Different unlinked transgene inserts can be present in the genome of one plant also as a result of the introduction of multiple insert copies by transformation of a single ModSeq (**Fig. 2**, left). From the analytical point of view, no difference can be made between ”multi-copy events” obtained by single transformation of a single ModSeq and GS produced by crossing, because the T-DNA is integrated in different loci which are physically unlinked (Holst-Jensen et al. 2006). The difference is in the phenotype: “multi-copy events” contain only one or a few but linked effective genes resulting in a single trait, while “stacked events” contain multiple traits. For this reason also we speak about single ModSeq stacks (SMS) for the first group and multiple ModSeq stacks (MMS) for the second, where both groups are examples of GS (**Fig. 2**).

This difference between stacking of genes or GS and stacking of traits or “events” is important in the frame of analytical detection and identification of GMOs. All stacked events are GS, however the opposite is not true. In this way, the definition of GS as subject of this deliverable, applies to all situations/cases where multiple, but not necessarily different, genes are inserted in the plant genome.

Taking into account this duality of the term “stacking” – stacking of events/traits versus stacking of genes – we can see also the discrepancy between legislatory and analytical requirements. Seen from the breeders’ point of view, in cultivation and research and for regulatory approval purposes (e.g. in the USA), stacking is interpreted in terms of combination of events/traits. Seen from the viewpoint of analytical detection, stacking is considered as the combination of genes. In regard to this, one could refer to “event stacking” as the presence of two or more different effect genes introduced and inserted in the host genome and leading, upon expression, to different new traits or properties of the plant. As such, multi-copy events or SMS (**Fig. 2**) would not be real “stacked events”.

Besides multiple single inserts of the same construct, other types of GS not considered so far are tandem repeat inserts and co-transformed partial constructs and rearrangements. Although multiple ModSeqs are present in such cases, as long as they concern (parts of) the same effective set of genes, i.e. the same transgenic trait, these cases would not to be considered as “stacked events” according to the definition in the previous paragraph. But they are still covered in the definition of GS (**Fig. 2**).

4 Cultivation and regulation of gene stacks

4.1 Global status of cGS cultivation

In 2006, herbicide tolerance deployed in soybean, maize, canola, cotton and alfalfa occupied 68% of the global biotech area, while the proportion of Bt cultivated plants was 19%. Commercial GS (cGS) conferring both herbicide tolerance (HT) and insect resistance (IR) traits combined by crossing, comprised 13% of the total global biotech area. The combination of HT and IR by cross-breeding was the fastest growing GM feature (30%) in 2006 (James 2006). In the USA, the largest adopter of biotech crops, stacked traits reached 39% of the cotton plantings and 15% of the maize plantings in 2006 (ERS, 2006). The cultivated GS biotech crops contain mostly double stacks traits, but in 2005, in the USA, a triple stack was deployed for the first time (James, 2005). **Table 3** (Annex 2) summarizes

the GS events produced by crossing of two or more transgenic events which have received authorization in different countries worldwide.

While in 2006 most common traits in biotech crops were (multiple) herbicide tolerance(s), (multiple) insect resistance(s) and the combination of both traits in one crop, expected for the next decade of biotech crop cultivations is the introduction of multiple genes involved in multiple metabolic pathways (metabolic engineering). While today, cross-hybridization, re-transformation, transformation with multigene cassettes and cotransformation are the main ways of stacking several traits, new multiple-trait stacking technologies have recently been announced. An example is the SmartStax™ technology from Monsanto and Dow (Monsanto, 2007), aiming at an eight-gene stack in corn and combining IR traits Herculex (2, Dow) and YieldGard (2, Monsanto) and HT-weed control systems RoundupReady and LibertyLink (Monsanto). Also, new multigene cassette assembling methods and plant transformation vectors e.g. polycistronic transgenes, are being deployed. (Halpin, 2005; Shrawat and Lörz, 2006).

4.2 Environmental and health risks associated with cultivation of GS

The cultivation area of the transgenic plants conferring more than one trait is progressively growing. This process might potentially have adverse effects on the environment and human and animal health.

EFSA guidelines for the environmental risk assessment of plants with combined events (EFSA, 2006a; 2006b) recommend to assess the potential environmental effects as result of the potential interaction between both (or more) introduced gene products. Points to consider in this evaluation are:

- Altered toxicity of the gene products and possibility to develop resistance in the target organisms;
- Enhanced toxicity to non-target organisms;
- Altered fitness of the GM plants and plants acquiring the transgene combination through gene flow;
- Enhanced capacity for gene flow and introgression;
- Altered effect in relation to biological diversity.

De Schrijver et al. (2007) identify a few potential risks that could arise as result of interbreeding of GM cultivars: change of level of expression of the introduced proteins compared to the GM parental lines; changed effect on the target and non-target organisms or cross resistance (in case of Bt toxins); environmental risks associated with increased invasiveness of the crop or its sexually compatible relatives; changes in farmland biodiversity. The authors emphasize on the need to perform specific field experiments before commercialization of GS depending on the crop on the one hand, and to establish a specific post-market monitoring scheme to study the potential adverse effects of their cultivation on the other hand.

At current, GS is limited primarily to traits like herbicide tolerance and insect resistance. Concerns exist mainly about the cultivation of crops conferring different Bt-toxin genes. The mode of action of Bt toxins could raise concerns about possible synergetic effects or mediating novel effects when combined with other substances (adjuvance) on target or non-target organisms (e.g. Esquivel-Perez & Moreno-Fierros, 2005) and cross-resistances are envisaged (De Schrijver et al., 2007). The risk of fast development of resistance of the target organisms to Bt-toxins is demonstrated in a model study. The simultaneous cultivation of single gene crops together with stacked gene crops introduces a risk for faster development of resistance to both Bt toxins than the cultivation of only the GS crop. In Australia only the gene stacked cotton line 15985 (Bollgard II) is currently allowed for cultivation. In the first two years after introduction of this line, the use of cotton lines containing the single Bt genes found in 15985 cotton, e.g. Cotton 531, was prohibited (Shelton, 2005).

Gene stacking in HT plants can have significant impact on the environment in a few ways: it can provide a source of HT genes to future crops growing in the same or neighboring fields; it may change the management practices and lead to changes in the herbicides used or crops cultivated; and may

make it necessary to take new measures to control the non-crop land populations that might contain HT genes.

4.3 Legal and regulatory aspects of GS

Legally, gene stacking is tackled differently in different jurisdictions. Because a commercial GS (cGS; see also Fig. 1 and section 4.4) can be the result of traditional plant breeding (however with transgenic parents), a cGS is not automatically subject to regulation in all jurisdictions, contrary to e.g. the individual parents of a cGS. Summarized, in the **USA** and **Canada**, stacks are considered as products from conventional breeding with a presumption of biosafety testing. Registration is only needed upon identification of a specific hazard, e.g. synergistic effects of combining various insecticidal proteins. In the **EU** to the contrary, each "new stack" is considered as a new GMO which needs to be assessed and approved, even though individual events may have market approval. Here risk assessment is focused on possible additional effects. The EFSA Guidelines only apply to stacks obtained by cross hybridization (see also 4.2). Between these two types of jurisdictions for stacks, **intermediate approaches** exist. For these registration is needed. However, the product benefits from the presumption of safety, and limited data showing that the product behaves as expected is the only major data requirement (unless a specific hazard is identified).

Furthermore, there is a distinction between release into the environment, and application in food/feed. In relation to labeling, this distinction is particularly relevant, because detection methods used to ensure compliance with labeling regulations have very significant limitations in relation to GS.

The main issue in all GM legislation is whether or not the GMO (product) is safe, so the first question in relation to GS is therefore if the jurisdiction considers that GS has the potential to result in an unsafe product, when the parent GMOs are considered safe. If this is the case, then separate risk assessment of the cGS is required. In some jurisdictions this is considered on a case-by-case basis, e.g. Australia, Brasil, Canada, China, New Zealand and USA (AgBios, 2007, CFIA, 2004; CMOA, 1996; CTNBio, 2005; FDA, 1992; OGTR, 2000; 2007), and whether the cGS formally becomes authorized or not may vary. For example, in the USA, a cGS is normally deregulated (authorized) if the parent GMOs are deregulated. However, the US Environmental Protection Agency (EPA) requires separate review of the safety of the cGS if the parental GMOs carry traits encoding biotoxins ("plant incorporated protectants") such as insecticides (EPA, 2001), reflecting that combinations of biotoxins may result in synergetic or adjuvant effects. In other jurisdictions advance notification prior to release is always required, e.g. Argentina, Canada, EU, Japan, Korea and the Philippines (AgBios, 2007; CFIA, 2004; EC, 2001; SAGPyA, 2003). Despite the recommendations in a recent discussion paper (OGTR, 2005), specific requirements for provision of detailed information permitting a safety assessment of the cGS are not included in the amendments of the Australian Gene Technology Regulations (OGTR, 2007).

The legal status of wild GS (wGS; see Fig. 1 and section 4.4) is, to our knowledge, not discussed specifically in any existing legislation, although it is to some part covered e.g. in relation to unintended dispersal of transgenic material. Furthermore, if a cGS is planted, progeny corresponding to each of the parental GMOs may be produced from the cGS. Consequently, authorization of a cGS should automatically imply authorization of the parental GMOs. Similarly, GMOs corresponding to segregated ModSeqs in the progeny of a MulEv should automatically have status as authorized if a MulEv is authorized. How such progeny is to be detected and quantified is uncertain unless each ModSeq is targeted.

4.4 Intended versus non-intended gene stacking

In the context of regulation, for stacked events it is useful to make a distinction between intended and non-intended gene stacking (**Fig. 1**). The former are deliberately produced and subject to authorization and commercial use while the second accidentally appears as a result of unintended hybridization on or around the cropping areas with low frequencies. Consequently, the impact of both groups will be different due to their different spreading in the environment. Unintended GS are formed only in

circumstances that allow close contact between transgenic plants carrying different transgene traits. A few scenarios for their formation and persistence can be envisaged:

1. Gene stacking on field: As a result of pollen flow from one transgenic plant to another transgenic plant GS seeds can be formed and they can remain in the soil and re-grow in the next cultivation period as volunteers. Alternatively farm saved seeds can be grown later.
2. Gene stacking around the field (in feral populations): unintended GS can form as a result of intraspecific crossing of transgenic plants persisting in feral populations out of the cropped land.
3. Undetected contamination in **seed production** lines: e.g. if farmers take seeds from own harvest or if seed production facilities do not apply sufficiently thorough testing, as recently demonstrated by the incidental presence of Bt10 maize and LL601 rice in commercial production the USA.

Formation and persistence of non-intended GS will depend on some factors specific for each crop:

- Biology of the plant – annual or perennial species.
- Reproductive system – self or cross pollinator; the gene flow via pollen dispersal will occur in cross pollinators (out-breeders) but not in obligate self-pollinators (in-breeders). Out-crossing rate in out-breeders will affect the frequency with which non-intended stacking occur.
- Fertility of the pollen.
- Pollen dispersal mechanism – e.g. dispersal with insects or wind may drastically affect transfer from adjacent plants or fields.
- Seed dispersal – in some species the gene flow occurs via seed dispersal.
- Volunteers persistence – the seeds of some species can persist in the soil and re-grow, whilst for other species seeds can not survive through the winter or compete in the environment.
- Ecology of the species – some species e.g. *Brassica napus* persist in populations out of the cropped land.
- Agricultural practices – depending on the agricultural practices the volunteers and out-crossing rates can be managed and the wGS can be prevented and the persistence of wGS can be minimized.

As we mentioned above, the probability of development of non-intended GS is crop dependent. However, persistence may also be affected by other factors, including selection pressure and genetic drift. Hereafter we summarize the crop specific biological characteristics and agricultural practices that can lead to occurrence of non-intended GS for three major transgenic crops: maize, oilseed rape, and cotton.

4.4.1 Maize *(Zea mays* L.*)*

Zea mays is a monoecious (both genders on same plant) annual grass (family Poaceae, i.e. monocot) that propagates through seed produced predominantly from cross pollination. Maize pollen is very promiscuous, lands on any silk and germinates immediately after pollination and completes fertilization after 24 hours (OECD, 2003). In the EU most of the varieties are hybrids. The production fields of hybrid seeds are more susceptible to pollen flow than the crop production fields. Pollen flow depends on the landscape conditions: size of the field and presence of natural barriers. To prevent pollen contamination of neighboring fields, measures are taken such as isolation distances, buffer zones, use of varieties with different flowering times. Under EU conditions the development of wGS can occur via pollen flow between adjacent transgenic fields. However, in the EU the cultivation of GM maize is currently limited to event MON810 only.

Gene flow in maize can occur between maize and other wild species like teosinte only in the territories where such wild relatives are present (e.g. teosinte in Mexico and Guatemala). Maize can also make inter-specific crosses although this is extremely unlikely. During its domestication maize has lost the ability to survive in the wild and needs human interference to effectively disperse the seeds, although birds and rodents may also spread seed. The plants are incapable of sustained reproduction out of the domestic cultivation and are not invasive in natural habitats.

Maize is an annual plant that dies out in the winter, some seeds can overwinter but usually after harvest they do not re-grow, propagate and give seeds. Under most European conditions the volunteers are easily managed by the agricultural practices. Gene flow through seed dispersal is possible where the farmers save seeds and use them in next propagation period (e.g. in Mexico; Bellon and Berthaud, 2004). In Mexico for instance maize GS conferring combined (two or more) traits such as herbicide tolerance (CP4 EPSPS) and insect resistance (Bt-*Cry9C*, Bt *Cry 1Ab/1Ac*) have been reported (Quist & Chapela, 2001; see also Cleveland & al., 2005). This concerned farms with small scale production where seeds were saved and later cultivated.

4.4.2 Oilseed rape *(Brassica napus* L.*)*

Oilseed rape belongs to the family Brassicaceae (crucifers, dicots) and is primarily self pollinating. The pollen can be transferred from plant to plant through physical contact between the neighboring plants and by wind and insects. Pollinating insects play a major role in the pollination (OECD, 1997).

After harvest some seeds can escape and overwinter and germinate either before or after the seeding of the succeeding crop. In some cases the volunteers can give significant competition to the seeded crop, which can be avoided by using agricultural practices such as crop rotation and suitable soil treatment. *Brassica napus* can easily make crosses with related species which makes the introgression of transgenes to wild species probable. However the formation of fertile interspecies hybrids depends on the donor of the pollen (OECD, 1997).

Brassica napus and its progenitors are 'primary colonizers' – species that are first to take advantage of the disturbed land habitats, where they compete for space with plants of similar types. In non-natural ecosystems e.g. along roadsides, transportation routes, industrial or waste sites there is a potential for ever-present populations because of the colonizing nature of the plant. The formation of feral populations will depend on the competitiveness with other species and on the climatic conditions. For instance feral populations of oilseed rape along road sides and ports occur in Japan (Saji et al., 2005), also frequently in UK, but are rare in Canada (Orson, 2002). Large amounts of *B. napus* plants can persist as a volunteer and may cause volunteer weed problems in crop lands with poor management practices where the *B. napus* seeds have not been properly harvested.

Gene stacking in oilseed rape can occur via pollen flow from neighboring transgenic fields or crossing of transgenic plants in feral populations.

Spontaneous sequential cross pollination between two herbicide tolerant oilseed rape varieties resulted in evolution of multiple herbicide resistance in Canada (Hall et al. 2000). Stacking of Roundup Ready (RR) and Liberty Link (LL) volunteers has been observed in 11 RR fields: 1% over the field edge and varying between 0.1 and 0.2 % at distances of 50 to 400 m within the crop. Surviving crops are less than 0.5 to 1 plant per m^2 and similar numbers of GS volunteers can occur via seed dispersal (Beckie, 2001). A similar rate of gene flow is reported in the UK (Ingram, 2000).

GM oilseed rape plants show fitness and yield comparable with conventional seed varieties (Fredshavn et al. 1995). Multiple herbicide tolerant volunteers displayed fitness similar to that of single herbicide tolerant plants or non-GM oilseed rape plants. (Simard et al. 2005). Depending on the nature of the transgenic variety the fitness of the herbicide tolerant volunteers might vary (Messéan et al. 2007). Selection pressure, e.g. due to agricultural and horticultural practice may favor GM volunteers, and GS may therefore have particularly high fitness under some circumstances. Admixture of GM oilseed rape seeds can occur after 5 year crop rotation at frequencies much higher than the European labeling threshold (Messéan et al. 2007). Volunteers are a major source of gene stacking (Messéan et al. 2007) and this process seems to be inevitable and almost impossible to prevent it as it is shown also from the Canadian studies (Beckie et al. 2001, Beckie et al. 2003).

However, agricultural practices like introduction of isolation distances (e.g. 50 m distance between HT plants that are not varietal associations in SCIMAC) will reduce its occurrence (Orson, 2002) The multiple and single herbicide tolerant oilseed rape plants are not less sensitive to herbicides usually used to control volunteers and susceptible to them in very early stage (Beckie et al. 2004). Use of

appropriate herbicides could control the presence of volunteers. The choice of suitable transgenic and conventional varieties is an important factor that can reduce the persistence of transgenic volunteers in the soils and subsequently prevent gene stacking on field (Messéan et al. 2007).

Controlling the stacked HT oilseed rape volunteers might not be significant in certain circumstances, e.g. in the UK the use of glufosinate is presently very limited and stacking of glufosinate and glyphosate tolerance genes will have no additional practical significance. The stacked HT volunteers could be a problem when they need to be controlled in other HT crops such as maize and sugar beet which could change the cultural and chemical practices (Orson, 2002).

In Japan, where no oilseed rape is cultivated, GS occurs in feral populations (Saji et al., 2005, Aono et al., 2006). The origin of the wGS is not clear but it could occur either as a result of spontaneous cross hybridization or via seed dispersal of imported stacked seeds (Aono et al., 2006).

The HT oilseed rape plants might persist in feral populations for a considerable time, but at the moment there are no data available that can prove long-term persistence of HT oilseed rapes in non-cropped lands. However, the cultivation of HT oilseed rape might increase the desire to control the feral populations (Orson, 2002).

4.4.3 Cotton *(Gossypium hirsutum* L.*)*

Cotton is a perennial shrub, or in the wild occasionally a small tree, that belongs to the family Malvaceae (dicots) and grows to about 1.5 m in height (max 5 m). Commercially, cotton is cultivated as an annual plant, with destruction of plants after harvesting the fruit for seed and fiber. Cotton is the most important non-food crop in the world and is also a target to genetic modification. In the EU cotton is mainly cultivated in Greece and Spain. Wild cotton may be found in Southern USA and Central America and on Pacific islands (Messean et al., 2006).

Cotton is a self-pollinator. The pollen is heavy and unlikely to be dispersed by wind, but pollen dispersal can be mediated by insects. In Andalusia for instance the outcrossing rate is estimated at 1% (Messean et al., 2006). In Australia the outcrossing rate between fields with transgenic and non-transgenic cotton is estimated to be 0.9% at distances below 10 m (Llewellyn and Fitt, 1996).

Cross-pollination of one *G. hirsutum* plant to another, mediated via an insect pollen vector, is the most likely means by which cotton genes could be dispersed in the environment. In Australia, gene transfer between adjacent *G. hirsutum* individuals occurs, albeit at relatively low frequencies.

Gene flow from cultivated *G. hirsutum* to feral cotton populations is also possible and viable seeds would be generated if it occurred. The likelihood of this is remote (OGTR, 2002).

Cotton is spread in the environment via seed dispersal. Cotton seeds can be dispersed also along side roads close to the production areas and lead to occurrence of volunteers which can reach maturity and persist for a long time. The volunteers can be destroyed by applying roadside management practices. There is no data of wGS occurrence in cotton cultivation areas or feral populations.

5 Detection of gene stacks

5.1 Specific issues related to GS identification and quantification

If the sole purpose of testing is to determine if GM material is present, then it may not be necessary to test for stacking. However, if for instance the GS is legally considered distinct from the parental GMOs and it is necessary to discriminate between authorized and non-authorized GM material, then identification of stacked material may immediately become necessary. Specific detection and quantification methods are available for most commercialized UniEv GMOs, and these methods may be used to identify and quantify cGS. However, these methods are limited by their lack of ability to

discriminate between a mix of two separate GMOs (the parents of the stack) and a mix of non-GM and the cGS (**Fig. 3)**.

We will separately discuss the issues of GS identification and quantification, with special focus on how to distinguish between authorized and non-authorized GS.

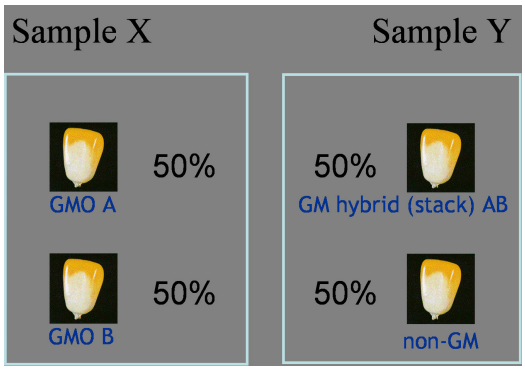


Figure 3. A 50:50 (%) mix of non-GM and a double stacked hybrid (AB) can only be distinguished from a 50:50 (%) mix of the two parental events A and B if the A and B alleles can be shown to have a non-indepent distribution (co-occurrence). This is easily achieved on a single seed/kernel, but not in a processed product like flour.

Firstly, labeling requires identification. As stacking reflects multiple events and/or genes inserted in one genome, identification comes down to unambiguous, unique, transformation event-specific identification.

- With the current EU legislation, the authorization of a stacked event obtained by crossing single events goes through separate authorization procedures (e.g. double stack: two validated event-specific methods needed), regardless whether or not the individual lines have/had been authorized.
- It is impossible to define a unique molecular marker for a StaEv, that will be vertically transmitted along with the ModSeqs. As a consequence, the detection method for a stacked event is a set of detection methods for each individual ModSeq. Related to this is the choice of (certified) reference materials to use for detection of GS: If the above is sufficient, then individual, single-event CRMs can be used for the purpose of identification of GS.

Secondly, labeling on the basis of a threshold implies a need for quantitative methods. Many countries require the labeling of GM products if the GM share of a single ingredient exceeds a specific threshold: e.g. 0.9 % in the European Union, 1% in Australia and New Zealand, 3%, in Korea and 5% in Japan and Indonesia (Carter and Gruere, 2003). Labeling of GM material is required provided that the quantity exceeds such a defined threshold and that the material is derived from authorized GMOs. For unauthorized GMOs the tolerance is much lower. In some jurisdictions (e.g. EU) there is zero tolerance, in others the tolerance level is not necessarily well defined. When the GM content of a harvest or any GM product is being measured, GS may have a strong influence on the measured GM content (see Holst-Jensen et al., 2006 for more details). Compared to a weight or particle (e.g. seed/kernel) based approach, a holoploid genome based approach may result in overestimates of the GMO quantity, since the presence of e.g. two ModSeqs in a single holoploid genome would yield an estimated GMO concentration of 200%. On the other hand a hemizygous single UniEv derived GM maize seed will yield an estimated GMO concentration of only 40-60%. In contrast, if seed is the prevailing unit, the same seed is deemed 100% GM. Thus, there will be cases where the measured GMO content is lower and cases where it is higher depending on the applied approach. The main difference, according to Holst-Jensen et al. (2006) is whether the approach applied is consistent and coherent or not.

- It is presently not clear if authorized cGS need to be quantified separately from the parental GMOs in those jurisdictions where GM material labeling is required. However, farmers need to know the

characteristics of the seeds they sow, so consequently seed traders need to be able to identify and quantify GS in the seeds. The International Seed Testing Association (ISTA) has chosen a "performance based approach" to GMO testing of seeds (ISTA, 2007). Unfortunately, this does not give clear guidelines on how to discriminate between stacked and non-stacked seeds.

- As pointed out by Holst-Jensen et al. (2006) a full implementation of haploid genome equivalent (HGE) based measurement will have clear consequences for the resulting GM quantity determination since GS contain more ModSeqs than UniEvs. While this is in principle a matter of political decision, in reality it is a matter of scientific consistency. Analytical methods can only measure analytes, and these can, within certain limits, be traced throughout the food/feed chain. Seed or mass based units, while more easily appreciated by politicians and laymen, are not traceable after processing that involves e.g. grinding of the seed. Consequently, from a scientific point of view the only traceable and coherent unit is the HGE, despite political and historically based opposition. One remaining point for clarification is linked with the definition of "haploid genome". Haploid genome refers to both "monoploid chromosome complement" (chromosome number x e.g. $x = 7$) and "holoploid chromosome complement" (whole chromosome complement with chromosome number n e.g. $n = 3x = 21$) (Greilhuber et al., 2005).
- Another important issue is to consider in how far the distinction in types of gene/event stacks, as described above, needs to be made in relation to quantification of the stacks. Should a quantitative analysis of a MulEv (obtained e.g. by co-transformation) be treated differently from a quantitative analysis of a StaEv (obtained by crossing single events), if the ModSeqs in both events are the same? As explained before, the different ModSeqs (inserted genetic constructs) are physically unlinked, thus per definition and from analytical viewpoint, there is no difference (Holst-Jensen et al., 2006). From a detection point, it was argued that the most coherent approach would be to treat StaEvs and MulEvs equally, and that the GM quantity for all GM materials should be measured and reported with reference to quantity of ModSeqs relative to haploid genomes per species.
- We could extend this issue to the question: What is the impact on quantification of the way of transformation (e.g. only nuclear DNA is transformed versus transformation of extranuclear DNA) and the way of production/origin of the GS (e.g. MulEv versus StaEv but also, MulEv versus single-transformed multi-copy event, see **Fig. 1** and **Fig. 2**). Analytically it may also be impossible to distinguish between wGS and cGS. **Table 4** serves to illustrate the analytical challenge. Analytically, based on DNA, there is a significant difference between measuring ≥ 3 ModSeqs (Bt176), 2 ModSeqs (stacked T25 x MON810) and 1 ModSeq (T25 or MON810) per mass or particle unit. It may be desirable to clarify in regulations how GS shall be treated in a more coherent manner. We recommend to follow Holst-Jensen et al. (2006). This is also in line with the core of EC recommendation 787/2004 (EC, 2004b).

Table 4. Comparison of "unique events" (UniEv), "stacked events" (StaEv) and "multiple events" (MulEv) in relation to their DNA content.

Type of GMO	OECD unique identifier	Popular name of GMO	ModSeq	Copy no in the GMO[a]
UniEv	ACS-ZMØØ3-2	T25 maize	PAT	1
UniEv	MON-ØØ81Ø-6	MON810 maize	CryIAb	1
StaEv	ACS-ZMØØ3-2 x MON-ØØ81Ø-6	Stacked T25 x MON810 maize	PAT CryIAb	11
MulEv	SYN-EV176-9 (176)	Bt176 maize	PAT CryIAb	≥1[b] ≥2[b]

[a] The copy number in the GMO corresponds to the number of analytes contributed to a DNA based analytical test per haploid GM genome of the GMO. Protein content may vary, due to differences in expression levels between lines, individual plants and on the basis of abiotic conditions. In bioassays, the trait is only detectable if the expression level is sufficiently high to induce an observable biological effect, and if biocide has the desired effect on the target organism.

[b] Present information on the AgBios database (AgBios, 2007) does not specify the number of copies present in the GMO. Earlier reports from the developer states that each gene is present in 2-5 copies in the transformed plant genome. It is also possible that the number of copies varies between commercial seed lines on the market since variations of sequences have been observed, for instance between Pactol and Garona cultivars (Y. Bertheau, unpublished).

5.2 Existing methods for GS identification and quantification

As mentioned above, transformation event-specific detection methods exist for most UniEvs. Official, internationally validated PCR-based methods are available e.g. from the EC's GMO-CRL website (http://gmo-crl.jrc.it/). Application of the single UniEv methods for identifying the multiple events or genes in GS, has as limitation that distinction cannot be made between a mix of two separate GMOs (parents of a stack) and a mix of the cGS with non-GM (**Fig. 3**). The only possibility to distinguish mixtures of different events and stacked events (containing the same ModSeqs) is to analyze the single individual plant or fruit (seed, kernel, etc.).

- Akiyama et al. (2005) describe a seed-based, single-kernel analysis method based on grinding of individual grains (MON810, GA21, MON810 x GA21) and multiplex qualitative real-time PCR detection of SSIIb, P35S and GA21-construct in one tube. Individual kernels contain either one of the transgenes (single events) or both transgenes (StaEv MON810 x GA21), which can be distinguished based on amplification plots, end-point analysis (fluorophore emission intensities), or agarose gel separation of PCR products. This method could be used to analyze seed lots but it will require analysis of a large seed number. In regard to the mixed food and feed samples the detection of the stacked gene event would not be possible because the plant structures are disintegrated.
- Identification of stacked genes in seed pools was demonstrated by Allnut et al. (2006). These authors developed a protocol for seed testing combining a sub-sampling strategy with real-time PCR. The first phase of the protocol was a test of oilseed rape pools containing stacked event MS8xRF3 by real-time PCR using assays detecting the MS8, RF3 and BAR. The positive pools did not segregate, i.e. one and the same pool was positive for all three assays which is a certain indication that the events are stacked. In a real situation where both events were observed to segregate in the subsamples the interpretation would be that the two separate transgenic events are independent (not stacked) (Allnutt et al. 2006). Unfortunately, this approach can not be applied to samples where the stacked material is mixed with the parental GMOs.
- Besides DNA-based methods, protein-based methods can be considered helpful in the detection of cGS. Ma et al. (2005) describe the evaluation of protein-based methods to detect LL, Bt and the stacked Bt/LL events in seed and grain samples. The authors evaluated SDI and Envirologix lateral flow kits for Bt and LL traits. However, application of protein based assays can identify gene stacks only on single seeds. In other words, the limits are basically the same as for DNA, but in addition, if two constructs encoding the same protein are present they cannot be distinguished, and if the protein is not expressed then the protein test will be negative. An example of a GMO where this could be a problem is Bt176 maize where two different constructs containing the CryIA(b) gene are regulated by tissue specific promoters that are not meant to be active in the maize kernels.
- Kobilinsky and Bertheau (2004) developed a statistical approach for GMO detection by application of qualitative testing methods taking into account the cost of analysis. This method is not directly applicable to stacked events and another approach must be studied in this particular case. A detection kernel by kernel is already developed by Akiyama et al. (2005), but this method is too laborious and expensive. INRA is currently exploring the possibility to use a statistical model based on multinomial distribution to test for the presence of stacked events by examining group of grains with PCR techniques. A quantitative PCR detection approach is already studied by INRA. This approach needs another model than the qualitative case. A quantitative model is a more interesting challenge in statistical theory with problems in parameters estimation and maybe conditional probabilities. This is presently subject to further study in WP4, task T4.4 of the Co-Extra project.

5.3 Other remarks

Currently, all GM plants carry their ModSeq in the nuclear DNA. As a consequence, the inserted sequences are transferred to successive generations in a Mendelian manner. What has not been considered so far is the situation where the ModSeq is inserted in extranuclear DNA in organelles (e.g. chloroplasts, mitochondria). In this case, the transgenic DNA would be inherited mostly maternally

(uniparentally). Plastid transformation (chloroplasts) has been proposed as a strategy to contain the genetic modification and reduce potential unintended dispersal via pollen (Co-Extra WP1).

5.4 Exploring possibilities to detect GS in seeds

The possibilities to deploy existing approaches to detect GS produced by crossing of two transgenic events (MON810xT25) in seed pools will be further explored within this Co-Extra work package.

An approach combining the 3D sampling strategy described by Degrieck et al. (2005) and real-time PCR quantification will be developed. The 3D sampling approach will be optimized by e.g. automation at the level of pooling / crushing-grinding / real-time PCR detection in stead of gel electrophoresis based detection. The MON810 and T25 genome copies will be quantified by event-specific real-time PCR in single seeds (or pools of seeds) and estimation of transgene copy number ratios, clustering and modeling the data based on the genome copy number ratios of both events in single seeds. The possibilities to distinguish stacked gene events based on haploid genome numbers and the architecture of the maize kernel will be explored. This is based on the assumption that the transgene copies of MON810 and T25 will differ between the crosses depending on which event is the donor of the pollen. The difference in the copy number is a consequence of the existence of diploid embryo and triploid endosperm in the maize kernel (Trifa and Zang, 2004; Papazova et al., 2005; 2006). The created model will be tested on seed mixtures containing GS.

6 Conclusions

The following are our main conclusions and identified remaining gaps, future needs and recommendations:

- Definitions related to stacking: Given the different ways of transformation and production of stacked events, including classical breeding, as well as differences in global authorization and commercialization practices, we conclude that the definition of stacking is inconsistent. Stacking can primarily be explained in terms of either the stacking of traits/events, or the stacking of genes. This review has tried to summarize the different perceptions and existing terms and definitions related to stacking. Seen from production and commercialization, as well as regulatory and analytical detection points of view, a general terminology is proposed as depicted in Fig. 1 and in particular in Fig. 2.
- Cultivation practices of cGS and occurrence of wGS: Taking suitable measures to limit the gene flow between transgenic fields, the formation of GS can be limited and the persistence of GS volunteers can be minimised or practically avoided by application of suitable crop specific agricultural practices. However, in the EU currently no GS are cultivated, and the cultivation of transgenic plants is limited. Unintended GS can occur in feral populations as a result of seed dispersal during transportation. The occurrence of wGS in feral populations (mainly for oilseed rape) is still not sufficiently investigated. Measures to manage feral populations could be of more interest in the future to avoid persistence of wGS volunteers.
- Detection and quantification methods: Analytical methods to detect GS are primarily seed-based detection strategies, making use of event-specific PCR assays or protein assays. There is no single molecular or genetic marker that can be identified or used for GS detection. We believe that scientifically a consistent application of a haploid genome equivalent (HGE) based approach for detection, identification and quantification is the best alternative. However such an approach cannot be used to identify GS material in homogenous processed matrices unless the material is composed solely of one type of GS material.
- Regulation of GS at EU level: Within the EU, the regulations in place are very comprehensive with respect to defining the basis for identification and quantification of GM materials. The definition of "event" is essentially the definition of what to identify/quantify. Therefore, it is clear from this report that while many jurisdictions have very incomplete regulations, there is still some need for further clarification also in the EU regulations. Most importantly, and underlying the political decisions, is the question: Shall the regulations be scientifically coherent or not? Coherence means first that the EU must decide what the prevailing unit of measurement and expression throughout the food/feed chain is. Should it be:
    - seeds/kernels ?
    - masses ?
    - volumes ?
    - haploid (holoploid or monoploid ?) genome equivalents (HGE) ?
    - protein equivalents (mass, numbers or …) ?

  Secondly it means that the unit shall be applied consistently, without *ad hoc* exceptions. A consistent application of the HGE based unit of measurement and expression will imply that GS material will have a higher assigned GM content than non-GS material. The advantages of this approach will, in our opinion, clearly outweigh the drawbacks. In particular, this approach may permit predictions and traceability of the GM content of products all the way from seed to fork once the GM content of the seed is known, with the uncertainty parameters being limited to sources of contamination (e.g. pollen influx, volunteers in the fields and bad cleaning of transport, storage and production equipment and containers). Without a clearly defined and prevailing unit of measurement and expression for the entire food/feed chain including seeds, there will always be room for legal dispute between stakeholders applying different measurement/expression units. In our opinion, the only scientifically justifiable solution would be to formally adopt the HGE as the only legally correct unit of measurement/expression of GMO content throughout the food/feed chain. It should be mentioned though that the so called botanical impurities, e.g. soybean volunteers in maize fields may create a problem unless these are also included in GM determinations (see also EC, 2004c). Botanical impurities are subject to further study in task T4.4 (milestone 4.2.8) in WP4 of the Co-Extra project.

7 References

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ANNEX 1: Table 2. Overview of stacked gene events. Summary of the transgenic events that received authorization worldwide, their method of production, introduced ModSeqs, traits (effect genes) and their copy number and loci number. The summary is made based on data from AgBios data base (http://www.agbios.com/main.php), BATS reports (http://www.bats.ch/index.php), European Commission information on GMO authorization, legislation and alike (http://ec.europa.eu/food/food/biotechnology/authorisation/index_en.htm)

Event	Method of production/	Traits	Introduced effect genes	Effect genes copy number	Loci number
Maize					
Introduction of one T-DNA insert (ModSeq) containing linked effect genes					
Bt11	Direct DNA transfer	LRes HT(Glu)	Cry1Ab pat	1	1
676	Particle bombardment	MS HT(Glu)	Dam pat	1 2	>1?
678	Particle bombardment	MS HT(Glu)	Dam pat	3 2	>1?
680	Particle bombardment	MS HT(Glu)	Dam pat	4 1	>1?
TC1507	Particle bombardment	LRes HT(Glu)	Cry1Fa2 pat	≥1 1	1
Mon88017	*Agrobacterium* mediated transformation/1	LRes HT(Gly)	Cry3Bb1 EPSPS	1	1
MON89034	*Agrobacterium* mediated transformation	LRes	Cry1A.105 Cry2Ab2	1	1
DAS-062758	*Agrobacterium* mediated transformation	LRes HT(Glu)	Cry1F bar	1	1
DAS59122	*Agrobacterium* mediated transformation	DLRes HT(Glu)	Cry34Ab1 Cry35Ab1 pat	1	1
Simultaneous introduction of two T-DNA inserts conferring different effect genes					
Bt176	Co-transformation via particle bombardments	LRes HT(Glu)	Cry1Ab bar	>2	>2(linked)
CBH351	Co-transformation by particle bombardment	LRes HT(Glu)	Cry9C bar	1 4	1
DBT418	Co-transformation via particle bombardment	LRes HT(Glu)	Cry1A(c) bar	2 1	1
Mon801	Co-transformation via particle bombardment	LRes HT(Gly)	Cry1Ab CP4 EPSPS	2 2 and 1 partial	2
Mon802	Co-transformation via particle bombardment	LRes HT(Gly)	Cry1Ab CP4 EPSPS	1 1	1
Mon809	Co-transformation via particle bombardment	LRes	Cry1Ab	2	1

		HT(Gly)	CP4 EPSPS	2	
MON832	Co-transformation via particle bombardment/2	LRes HT(Gly)	Cry1Ab CP4 EPSPS	0 2	1
MS3	Co-transformation	MS HT(Glu)	Barnase barstar bar	1	1
MS6	Co-transformation	MS HT(Glu)	Barnase bar	1	1
Sequential introduction of more than one effect gene					
ACSZM003- 2x MON810	F1 hybrid of T25 and Mon810	LRes HT(Glu)	Cry1Ab pat	1 1	2
DAS1507x MON603	F1 hybrid NK603 and TC1507	LRes HT(Glu) HT(Gly)	Cry1Fa2, PAT CP4 EPSPS	≥1 1 1	2
MON603x MON810	F1 hybrid NK603 and Mon810	LRes HT(Gly)	Cry1Ab CP4 EPSPS	1 1	2
MON863x MON810	F1 hybrid of Mon 863 and Mon 810	DLRes	Cry1Ab Cry3Bb1	1 1	2
MON863xMON810x MON603	F1 hybrid of stacked line Mon863XMon810 and NK603	DLRes HT(Gly)	Cry1Ab Cry3Bb1 CP4 EPSPS	1 1 1	3
GA21 x MON-810	F1 hybrid of GA21 and Mon810	LRes HT(Gly)	Cry1Ab CP4 EPSPS	1 1	2
NK603 X Mon863	F1 hybrid of Mon863 and NK603	DLRes HT(Gly)	Cry3Bb1 CP4 EPSPS	1 1	2
TC1507X AS59122	F1 hybrid of TC1507 and DAS59122	TLRes HT(Glu)	Cry1Fa2 Cry34Ab1 Cry35Ab1 pat	≥1 1 2 1	2
DAS59122X NK603	F1 hybrid of DAS59122 and NK603	DLRes HT(Gly) HT(Glu)	Cry34Ab1 Cry35Ab1 CP4 EPSPS pat	1 1 1 1	2
DAS59122X TC1507 X NK603	F1 Hybrid of DAS59122xTC1507 and NK603	TLRes HT(Glu) HT(Gly)	Cry1Fa2 Cry34Ab1 Cry35Ab1 pat CP4 EPSPS	≥1 1 1 2 1	3
MON810 X LY038	F1 hybrid of MON810 and LY038	LRes Lys	Cry1A cordapA	1 1	2

MON810XMON88017	F1hybrid of MON810 and MON88017	CRes LRes HT(Gly)	Cry1A Cry3Bb1 CP4 EPSPS	1 1 1	2
Bt11 X GA21	F1 hybrid of Bt11 and GA21	LRes HT(Glu) HT(Gly)	Cry1Ab Pat EPSPS	1 1 1	2
MON89034XNK603	F1 hybrid of MON89034 and NK603	LRes HT(Gly)	Cry11.105 Cry2Ab2 CP4 EPSPS	1 1 1	
MON89034X MON88017	F1 hybrid of MON89034 and MON88017	LRes CRes HT(Gly)	Cry11.105 Cry2Ab2 Cry3Bb1 CP4 EPSPS	1 1 1 1	2

Cotton					
Introduction of one T-DNA insert (ModSeq) containing linked effect genes					
19-51	*Agrobacterium* mediated transformation	HT(sulf)	Chimeric S4-HrA	2	1
531, 757, 1076	*Agrobacterium* mediated transformation	LRes	Cry1Ac	2	2
DAS-24236-5 (281-24-236)	*Agrobacterium* mediated transformation	LRes HT(Glu)-SM	Cry1F pat	1 1	1
DAS-21023-5 (3006-210-23)	*Agrobacterium* mediated transformation	LRes HT(Glu)-SM	Cry1Ac pat	1 1	1
23-198	*Agrobacterium* mediated transformation	FAC	Bay TE	3	1
23-18-17	*Agrobacterium* mediated transformation	FAC	Bay TE	15	5
Falcon GS/40/90	*Agrobacterium* mediated transformation	HT(Gly	pat	2	2
PHY14, PHY35 PHY36	*Agrobacterium* mediated transformation	HT(Glu) MS	Bar Barnase barstar	No information available	No information available
HCN92 (Topas 19/2)	*Agrobacterium* mediated transformation	bar	pat	2	1
Sequential introduction of more than one effect gene					
15985	Re-transformation via particle bombardment	DLRes	Cry2Ab Cry1Ac	1 2	1 1
DAS2103-5XDAS24236-5	F1 hybrid DAS2103-5 and DAS24236-5	LRes HT(Glu)	Cry1F, Cry1Ac pat	1 1 2	2
DAS-21023-5xDAS-24236-5 x MON-01445-2	F1 hybrid of DAS2103-5X DAS24236-5 and MON-01445-2	LRes HT(Glu)-SM? HT(Gly)	Cry1F Cry1Ac pat CP4 EPSPS	1 1 2 1	3
DAS-21023-5xDAS-	F1 hybrid of DAS2103-5xDAS24236-5 and	LRes	Cry1F	1	3

24236-5 x MON-88913	MON-88913	HT(Glu)-SM? HT(Gly)	Cry1Ac pat CP4 EPSPS	1 2 1	
MON-15985-7xMON-01445-2	F1 hybrid of MON-15985-7 and MON-01445-2	LRes HT(Gly)	Cry1Ac Cry2Ab CP4 EPSPS 	>1 1 1	3
MON-00531-6xMON-01445-2	F1 hybrid of MON-00531-6 and MON-01445-2	LRes HT(Gly)	Cry1Ac CP4 EPSPS	1 1	2
LL25xBgII Cotton	F1 hybrid of ACS-OS002-5 x MON-15985-7	LRes HT(Glu)	Cry1Ac Cry2Ab bar 	1 1 1	2
MON15985 x MON88913	F1 hybrid of MON15985 x MON88913	LRes HT(Gly)	Cry1Ac Cry2Ab CP4 EPSPS 	>1 1 1	3
Canola					
Sequential introduction of more than one effect gene					
MS1xRf1 (PGS1)	F1 hybrid of MS1 and RF1	HT(Glu) MS	Bar Barnase barstar	1 1 1	2
MS1xRf2 (PGS2)	F1 hybrid of MS1 and RF2	HT(Glu) MS	Bar Barnase barstar	1 1 1	2
MS8xRf3	F1 hybrid of MS8 and RF3	HT(Glu) MS	Bar Barnase barstar	1 1 1	2
Chicory					
Introduction of one T-DNA insert (ModSeq) containing linked effect genes					
RM3-3, RM3-4, RM3-6	*Agrobacterium* mediated transformation	HT(Glu) MS	Bar Barnase	1 1	1

Potato					
Introduction of one T-DNA insert (ModSeq) containing linked effect genes					
RBMT21-129, RBMT21-350, RBMT22-082	*Agrobacterium mediated transformation*	BRes VRes HT(Gly)	Cry3A Replicase from PLRV CP4 EPSPS	*1* *1* *1*	*1*
Sequential introduction of more than one effect gene					
RBMT15-101, SEMT15-02, SEMT15-15	retransformation	BRes VRes	Cry3A CP PVY	1 1	*1*

LRes= lepidopteran pests resistance
CRes= coleopteran pests resistance to
DLRes= double lepidopteran pests resistance
TLRes= triple lepidopteran pests resistance
EL/CRes=enhanced resistance to lepidopteran or/and coleopteran pests
HT(Glu)=tolerance to gluphosinate ammonium based herbicides
HT(Gly)=tolerance to glyphosate based herbicides
HT(sulf)=tolerance to sulfuronyl herbicides
Lys= enhanced lysine level
MS=male sterility/fertility restorer
SM=selectable maker
FAC=modified fatty acid content
BRes= resistence to collorado beatleCollorado beetle
VRes= virus resistance

ANNEX 2: Table 3. Authorization status of GS produced by combining two single transgenic events

Crop/event	Regulatory approvals	Proposed use
	Maize	
ACSZM003-2x MON810	Japan	Food/feed
	USA*	Food/feed, environment
DAS1507x MON603	Japan,	Food/feed, environment
	Korea	Food
	Philippines	Food/feed
	EU	Food/feed
	USA*	Food/feed, environment
MON603x MON810	Japan	Food/feed
	Korea	Food
	Mexico	Food
	Philippines	Food/feed
	EU	Food/feed
	USA*	Food/feed, environment
MON863x MON810	Japan	food/feed, environment
	Korea	Food
	Philippines	Food/feed
	EU	Feed
	USA*	Food/feed, environment
MON863xMON810x MON603	Japan	food/feed, environment
	Philippines	food/feed
	EU**	
	USA*	Food/feed, environment
GA21 x MON-810	Japan	Food/feed
	Korea	Food
	Philippines	Food/feed
	South Africa	Food/feed
	EU	Food/feed
	USA*	Food/feed, environment
NK603 X Mon863	Japan	Food/feed
	Korea	Food
	Mexico	Food
	Philippines	Food/feed
	EU	Food/feed
	USA*	Food/feed, environment
TC1507X AS59122	Japan	Food/feed
	Korea	Food
	EU**	
	USA*	Food/feed, environment
DAS59122X NK603	Japan	Food/feed
	Korea	Food
	Philippines	Food/feed
	USA*	Food/feed, environment
DAS59122X TC1507 X NK603	Japan	Food/feed
	Korea	Food
	USA*	Food/feed, environment
MON810 X LY038	Philippines	Food/feed
	USA*	Food/feed, environment
MON810XMON88017	Japan	Food
	Philippines	Food/feed
	USA*	Food/feed, environment
Bt11 X GA21	Korea	Food
	USA*	Food/feed, environment
MON89034XNK603	EU**	Food/feed, environment
	USA*	
MON89034X MON88017	EU**	Food/feed
	Cotton	
DAS2103-5XDAS24236-5	Australia	Food
	Japan	Food/feed
	Korea	Food
	Mexico	Food
	USA	Food/feed, environment
	EU – pending authorization	

DAS-21023-5xDAS-24236-5 x MON-01445-2	Japan	Food/feed
	Mexico	Food
	USA*	Food/feed, environment
DAS-21023-5xDAS-24236-5 x MON-88913	Japan	Food/feed
	USA*	
MON-15985-7xMON-01445-2	Australia	Environment
	Japan	Food/feed
	Korea	food
	Philippines	Food/feed
	EU**	Food/feed
	USA*	Food/feed, environment
MON-00531-6xMON-01445-2	Australia	Environment
	Japan	Food/feed
	Korea	Food
	Mexico	Food
	Philippines	Food/feed
	EU	Food/feed
	USA*	Food/feed, environment
LL25xBgII Cotton	Japan	Food
	EU**	
MON15985 x MON88913	Australia	Environment
	Japan	Food/feed
	Philippines	Food/feed
	USA*	Food/feed, environment
Canola		
MS1xRf1 (PGS1)	EU	Food/feed, marketing
	Australia	Environment, food/feed
	Canada	Environment, food/feed
	China	Food/feed
	Japan	Environment, food/feed
	Korea	Food
	USA	Environment, food/feed
MS1xRf2 (PGS2)	EU	Food/feed
	Australia	Environment, food/feed
	Canada	Environment, food/feed
	China	food/feed
	Japan	Environment, food/feed
	Korea	Food
	USA	Environment, food/feed
MS8xRf3	EU	food/feed, marketing
	Australia	Environment, food/feed
	Canada	Environment, food/feed
	China	food/feed
	Japan	Environment, food/feed
	Korea	Food
	USA	Environment, food/feed

*USA approvals for the parental lines

** under authorization under EC/1829/2003