








## Gene stacking as a strategy to confer characteristics of agronomic importance in plants by genetic engineering

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**ABSTRACT:** *Gene stacking refers to the introduction of two or more transgenes of agronomic interest in the same plant. The main methods for genetically engineering plants with gene stacking involve (i) the simultaneous introduction, by the co-transformation process, and (ii) the sequential introduction of genes using the re-transformation processes or the sexual crossing between separate transgenic events. In general, the choice of the best method varies according to the species of interest and the availability of genetic constructions and preexisting transgenic events. We also present here the use of minichromosome technology as a potential future gene stacking technology. The purpose of this review was to discuss aspects related to the methodology for gene stacking and trait stacking (a gene stacking strategy to combine characteristics of agronomic importance) by genetic engineering. In addition, we presented a list of crops and genes approved commercially that have been used in stacking strategies for combined characteristics and a discussion about the regulatory standards. An increased number of approved and released gene stacking events reached the market in the last decade. Initially, the most common combined characteristics were herbicide tolerance and insect resistance in soybean and maize. Recently, commercially available varieties were released combining these traits with drought tolerance in these commodities. New traits combinations are reaching the farmer's fields, including higher quality, disease resistant and nutritional value improved. In other words, gene stacking is growing as a strategy to contribute to food safety and sustainability.*

**Key words:** *co-transformation, re-transformation, sexual crossing, transgene, minichromosomes.*

## Empilhamento gênico como estratégia para conferir características de importância agrônômica em plantas via engenharia genética

**RESUMO:** *O empilhamento gênico se refere a introdução de dois ou mais transgenes de interesse agrônômico na mesma planta. Os principais métodos de produção de plantas geneticamente modificadas com empilhamento gênico envolvem (i) a introdução simultânea, pelo processo de co-transformação, e (ii) a introdução sequencial de genes, pelos processos de re-transformação ou por cruzamento entre eventos transgênicos. Em geral, a escolha do melhor método varia de acordo com a espécie de interesse e a disponibilidade de construções genéticas e eventos transgênicos preexistentes. Também é apresentado aqui o uso da tecnologia de minicromossomos como tecnologia potencial de empilhamento gênico. O objetivo desta revisão é discutir aspectos relacionados à metodologia para o empilhamento de genes a combinação de características (obtida via empilhamento de genes de interesse agrônômico) via engenharia genética. Além de discutir, é apresentado uma lista de culturas e genes aprovados comercialmente que tem sido usado em estratégias de empilhamento e uma discussão sobre normas regulatórias. Um número maior de eventos com empilhamento de genes foi aprovado e liberado no mercado na última década. Inicialmente, a combinação das características de tolerância a herbicidas e resistência a insetos era a mais popular, principalmente em soja e milho. Recentemente, estas características combinadas com tolerância a seca nessas culturas foram liberadas comercialmente. Novas características combinadas estão entrando na lavoura, incluindo aumento da qualidade, resistência a doenças e aumento do valor nutricional. Em outras palavras, o empilhamento gênico está crescendo como tecnologia para contribuir para a segurança alimentar e sustentabilidade.*

**Palavras-chave:** *co-transformação, re-transformação, cruzamento, transgenes, minicromossomos.*

## INTRODUCTION

The use of genetically modified crops (GMC) presents numerous advantages to the producer and to the consumer, as realized in greater productivity, reduced use of chemicals, cultivation of plants in adverse environmental conditions and higher nutritional quality (RANI & USHA, 2013). The advantages offered by GMC resulted in this

technology having the fastest adoption rate in relation to any other innovation in modern agriculture. In 2018, the global area cultivated with GMC was 191.7 million hectares representing an increase of approximately 113 fold since 1996, the first year GMC were commercially planted. The continued increase in GMC implementation in agriculture resulted in economic and environmental benefits, health improvement and social gains (ISAAA, 2018).

The first transgenic plants released commercially were engineered for herbicide tolerance and this remains the predominant characteristic of transgenic crops. But, advances in genetic transformation technologies, as well as the explosion of genomic sequencing technologies, have facilitated the introduction of multiple genes and characteristics in a single variety using gene stacking strategies (LUNDRY et al., 2013). As a result, there has been an increase in the new development of crops with multiple genes in relation to those with single-gene engineered traits (LUNDRY et al., 2013; ISAAA, 2019).

Transgenic plants with combined characteristics (stacked traits) are those that have different characteristics in the same plant conferred by different genes introduced by genetic engineering. In 2018, GMC with stacked trait occupied 42% of the global biotech crop area, and were more prevalent in the United States and Brazil (ISAAA, 2018). One example of stacked traits soybean is the event MON87701 x MON89788, trade named Intacta™ Roundup Ready™ 2 Pro, that carries the *cp4 epsps* gene, conferring glyphosate herbicide tolerance, and the *cryIAc* gene, responsible for Lepidopteran resistance. In addition, plants are available with stacked genes conferring resistance to different insect pests and/or tolerance to herbicides by different mechanisms. The most popular stacked maize event in that category is the MON-89034 x DAS-01507 x MON-88017 x DAS-59122, trade name Genuity® SmartStax™. This event harbor the *cp4 epsps* and *pat* genes for glyphosate and glufosinate-ammonium herbicide tolerance and also carry the *cryIFa2*, *cry2Ab2* and *cryIA.105* genes for Lepidopteran insect resistance as well as the *cry35Ab1*, *cry34Ab1* and *cry3Bb1* genes for Coleopteran insects resistance (ISAAA, 2019).

In addition, the genomic revolution has allowed the analysis of how the introduction of groups of genes together affects biological systems. The multigene transfer technology allows researchers to achieve goals that were once impossible, such as importing a whole metabolic pathway and expressing entire multi-protein complexes (NAQVI et al., 2010). The major application of multigene transfer thus far has been the modification of metabolic pathways. One example is the “Purple Endosperm Rice” which has high anthocyanin contents and antioxidant activity in the endosperm by the introduction of eight anthocyanin-related genes (two regulatory genes from maize and six structural genes from *Coleus*) driven by the endosperm-specific promoters

(ZHU et al., 2017). Also, considerable progress has been made in molecular engineering in order to improve photosynthetic performance in C3 plants by overproducing C<sub>4</sub> photosynthetic enzymes. Many plants, including transgenic rice have been generated and intensively analyzed for this purpose (TANIGUCHI et al., 2008).

The growing population and emerging environment challenges demand the development of more productive crops, more resistant to pests and diseases and tolerant to many stressful threats such as high salt, drought, flood, freezing and adaptation to poor quality agricultural land. In addition, the need to save water in agriculture is compelling the development of crops more efficient in its utilization. The multigene engineering in plants can make those goals more feasible to achieve.

The purpose of this review was to discuss the stacking of genes as a strategy to confer characteristics of agronomic importance in plants by genetic engineering. The aspects related to the methodology of production of plants with gene stacking, events approved with combined characteristics and regulatory aspects are presented.

#### *Methods for gene stacking*

Gene stacking refers to the process of combining two or more genes of interest in the genome of a single plant. There are different ways of obtaining plants with gene stacking. The procedures used can be separated into two main groups: (1) simultaneous introduction methods; (2) sequential introduction methods.

#### *Simultaneous introduction methods*

These methods refer to the introduction of several genes in the same process of genetic transformation. This technique is described as co-transformation and can be separated into two subgroups according to the methodology used: (1) when all genes are present in the same plasmid it is called “co-transformation with single plasmid” and (2) when genes are in separate plasmids it is called “co-transformation with multiple plasmids” (FRANÇOIS et al., 2002).

Co-transformation with a single plasmid: This method can be performed by introducing a single genetic construction with all genes, each with its own promoter and terminator, in the same plasmid. This can be used for genetic transformation by bombardment or *Agrobacterium tumefaciens*, and all genes tend to be integrated together in the same plant genome site(s).

The main difficulty of this process is associated with the size of the T-DNA to be introduced in the plant and the engineering necessary to the gene building with many genes requiring the use of convenient restriction sites, promoters and terminators. Emerging methods of engineering and manipulating large constructs (10s of Kbs) are making this an easier process (COLLIER et al., 2018). However, when the same promoter is used repeatedly for multiple gene expression, the alteration in the gene expression and gene silencing become a problem (AGAPITO-TENFEN et al., 2014).

It is possible to use vectors of transformation with linked genes that are regulated by a single promoter. This type of gene construction allows the simultaneous expression of the genes of interest and avoids the repetitive use of the promoter (SUN et al., 2012). Linked genes can be separated by sequences called Internal Ribosome Entry Sites (IRES). The IRES are sequences within the mRNA that promote the beginning of the CAP independent translation (the CAP is the regular ribosome binding site at the 5' end of the mRNA molecule). The IRES allows the ribosomes to be recruited to a translation initiation codon regardless of the distance from the CAP-5' end of the mRNA. When these sequences are placed between two genes regulated by a single promoter they can mediate the translation of the second gene independent of the first one (FRANÇOIS et al., 2002).

Two wheat genes, *anticarrier Na<sup>+</sup>/H<sup>+</sup> (TNHXS1)* and *H<sup>+</sup>-pirofosfatase (TVPI)*, coding for transporters of vacuolar ions, were linked via IRES for the generation of a bicistronic construction regulated by the promoter 35S. The IRES sequence was used from the target plant, *Nicotiana tabacum*, corresponding to region 5' UTR of the *heatshock factor1 (NtHSF-1)* (GOUJIAA et al., 2012). The molecular analyses of the genetically transformed tobacco plants demonstrated the correct integration of the construction in the host plant genome and the transcription of the bicistronic mRNA. In addition, an increase in salinity tolerance and water stress conferred by the expression of the two transgenes was observed in relation to plants containing each of the genes separately (GOUJIAA et al., 2012).

Another strategy is to separate the genes by connecting peptides. In this case, the transcription of a single mRNA leading to the formation of precursor polyprotein, which is proteolically cleaved for the release of the individual proteins (FRANÇOIS et al., 2002, SUN et al., 2012). The genes *cryIAh*, which confers resistance to Lepidopteran insects, and

*mG2-epsps*, which confers tolerance to the herbicide glyphosate, were linked by a hybrid connection peptides 2A/LP4. The 2A connection peptide is derived from the foot-and-mouth disease virus (FMDV) which has self-cleaving activity. The LP4 has a site of recognition and cleavage by a protease and is derived from seeds of *Raphanus sativus* (SUN et al., 2012). The vectors containing the *cryIAh* and *mG2-epsps* linked genes were transferred to tobacco plants and the transgenic plants expressed both genes phenotype, better than using either the 2A or LP4 sequences separately (SUN et al., 2012). In a similar way, the *cry1Ab* and *cry2Ab* genes were simultaneously introduced in rice using 2A linked peptide from different virus. The 2A self-cleaving peptides expressed Cry1Ab and Cry2Ab at high levels in the transgenic plants, which became highly resistant to Lepidopteran pests (ZHAO et al., 2014).

The transplastomic, plastids transformation technique, method of multiple gene transformation allows the use of multi-gene constructions controlled by only one promoter. Due to the prokaryotic nature of the plastids gene expression machinery, it is possible to develop operon type gene constructions, where genes are regulated by a single promoter and expressed as a polycistronic mRNA (BOCK et al., 2014).

Transformation of plastids presents the advantages of high expression of the transgenes, integration of transgenes by homologous recombination, more stable gene expression due to the absence of gene silencing and other epigenetic mechanisms, the possibility of the construction of synthetic operons, and maternal inheritance (reviewed by BOCK et al., 2014). Despite the advantages, the efficient transformation of plastids with precise and well-adapted protocols is limited to only a few species (FRANÇOIS et al., 2002; BOCK et al., 2014).

Co-transformation with multiple plasmids: In this process, several constructions are made, each with one or a few genes, and each construct is inserted into a separate plasmid. The plasmids can be used in genetic transformation by bombardment or *A. tumefaciens* delivery. In the case of *A. tumefaciens*, each plasmid can be inserted into a strain of the *Agrobacterium* and all of them placed in contact with the target explant tissue for the transfer of the T-DNA or, alternatively, all plasmids are inserted into the same strain of *agrobacterium* (HALPIN, 2005).

One example of the co-transformation strategy was used to engineering the 'Golden rice'. Using *Agrobacterium*-mediated co-transformation, two T-DNAs, each harboring two genes, were introduced into rice, enabling the endosperm to



express a carotenoid biosynthetic pathway and produce  $\beta$ -carotene (provitamin A) (YE et al., 2000). The production of this vitamin A precursor in rice endosperm is expected to help to alleviate vitamin A deficiency in certain regions of the world.

The simultaneous introduction of several genes has the advantage that the genes are most commonly introduced in nearby regions in the genome. In this way, there is less probability of transgene segregation in the next generations (HALPIN, 2005). However, in some cases, the process of co-transformation with different plasmids is carried out with the objective of obtaining transgene insertion in different locations of the genome and thus obtaining segregation in the next generation aiming to the eliminate the marker gene (RAO et al., 2011). As an example, the transgenic rice line CoT23 expressing the rice *chitinase* gene (*chi11*) (SRIPRIYA et al. 2008), involved in fungal resistance, was subjected to a new process of co-transformation using two different plasmids, one containing the marker gene *hygromycin phosphotransferase* (*hph*) and the reporter gene *beta-D-glucuronidase* (*uidA*). The other plasmid contained the *tobacco osmotin* (*ap24*) gene also involved in the resistance to fungi. Using the *A. tumefaciens* co-transformation process, it was possible to obtain plants with transgenes inserted into different regions of the genome enabling the segregation of transgene and elimination of the marker and reporter genes in the next generation. In this way, it was possible to add only the *ap24* gene to transgenic rice line CoT23 expressing the *chi11* gene and providing a high level of resistance against *Rhizoctonia solani* (RAO et al., 2011).

The main advantage of the co-transformation process is the possibility of introducing multiple transgenes into the same transformation process. This process reduces the time used for the production of events containing multiple transgenes. When co-transformation with single plasmid was used, the main difficulty is associated with producing and manipulating large T-DNA inserts containing many genes. In the co-transformation with multiples plasmids the main difficult is the frequency with which two (or more) independent transgenes are both transferred and integrated in the same plant cell genome (FRANÇOIS et al., 2002).

#### Sequential introduction methods

The sequential introduction of the genes can be obtained by the (1) re-transformation processes or (2) by the sexual crossing between two or more transgenic events.

Re-transformation: this process involves the transformation of an already transgenic plant to insert a new characteristic. Re-transformation has the advantage that the stacking of transgenes is independent of sexual crossing. Using this method, it is possible to combine transgene characteristics in plants that are normally vegetative propagated or have difficulties in the production of viable gametes or seeds by sexual crossing (FRANÇOIS et al., 2002).

One of the difficulties of this process involves the marker genes. With each new transformation, the use of different marker genes facilitates the selection of the transformed plants. The most commonly used marker genes are the ones conferring resistance to herbicide like *pat* gene from *Streptomyces viridochromogenes* and *bar* gene from *S. hygrosopicus*, both conferring resistance to glufosinate-ammonium herbicide. Marker genes also can confer resistance to antibiotics as kanamycin (*npt-II* gene), spectinomycin (*aad* gene) and hygromycin (*hpt* gene). The availability of relatively few marker gene in addition to the presence of multiple marker genes or multiple copies of the same marker gene in the transgenic plant may not be of interest and end up hampering the regulatory process of these plants (HALPIN, 2005).

Despite the need for different modes of selection for each transformation event in the re-transformation process, it is still possible to use the same selection gene by increasing the dose of the antibiotic in the selection of the second transformation process. CINGEL et al. (2014) retransformed the Jelica potato cultivar using this strategy. They used the same marker gene and higher selection pressure in the second transformation round to stack genes for proteinase inhibitors oryzacystatins I and II (*oci* and *ocii* genes).

However, the use of the same marker gene implies a greater number of copies of this gene in the plant genome. However, strategies resulting in the subsequent elimination of the marker gene can be employed, since these genes are only necessary for recovering transgenic plant lines in laboratory and no longer required in mature plants in the field.

The integrated use of the processes of re-transformation and co-transformation is one of the alternatives for the gene marker elimination. The co-transformation of the genes of interest and the marker gene in different vectors makes possible the elimination of the marker gene by segregation of the progeny. In this way, the plant with the gene of interest and without the marker gene can be subjected to a process of re-transformation with the same marker gene of the first transformation (RAO et al., 2011).

With the recent advance of the gene editing technology of CRISPR/Cas9 (Clustered Regularly Interspaced Short Palindromic Repeat), undesirable genes can be mutated to a non-functional variant (gene silencing) and possibly even deleted. This system has basically two important components, a guide RNA (gRNA) designed to have about 20 nucleotides complementary to the gene to be edited, and a unique kind of endonuclease, Cas 9, that cuts the DNA matched by the RNA guide. The CRISPR/Cas9 ribonucleoprotein complexes system could be transiently expressed in the plant, in a DNA-free genome editing strategy (SVITASHEV et al., 2016), just to eliminate the marker gene.

One of the main drawbacks of the sequential gene introduction processes is that transgenes will be introduced into different loci, and the segregation of transgenes can occur in the next generation and therefore require time-consuming co-gene maintenance procedures (QUE et al., 2010). However, emerging genome editing techniques can be used in conjunction with re-transformation to insert genes into specific locations of the genome (YU et al., 2016). One of the methodologies that can be used are the Zinc finger nucleases (ZFN). The *pat* gene, from *S. treptomyces viridochromogenes*, which confers resistance to the herbicide glufosinate-ammonium, was inserted into maize genome flanked by the target sequences of the ZFN enzymes. Subsequently, the re-transformation was carried out with the *aryloxyalkanoate dioxygenase 1* (*aad1*) gene, from *Sphingobium herbicidovorans*, which confers resistance to the herbicide 2,4-D (2,4-dichlorophenoxyacetic acid) flanked by sequences recognized by the ZFN already inserted along with the *pat* gene. Approximately 5% of the events resulted in the insertion of the *aad1* gene adjacent to the *pat* gene. The resistance to both herbicides, 2,4-D and glufosinate-ammonium, co-segregation in progeny, demonstrated the link between the two independently inserted transgenes (AINLEY et al., 2013).

Sexual crossing: most of the events with gene stacking approved by 2019 (ISAAA, 2019) were obtained by sexual crossing of two or more events. One of the main advantages of this method was flexibility. The use of previously tested and approved events and the possibility of adapting the characteristics of the new plant according to the needs of the market represented a great advantage (QUE et al., 2010). In some countries, such as the United States, Canada and Australia events resulting from the sexual crossing between previously tested

and approved events do not require new regulations, provided there is no evidence of possible interactions between proteins. In many cases, even if new crossing events require further regulatory approval, the process becomes simpler and faster due to prior evaluation and regulation of the donor plants (PILACINSKI et al., 2011).

The method used to obtain gene stacking via sexual crossing was technically simple. It involved only the transfer of pollen from one parent to the female reproductive organ of the other. However, it cannot be used in vegetative propagated plants (FRANÇOIS et al., 2002). Most maize commercialized events were obtained by sexual crossing between transgenic events like the TC1507 × 59122 × MON810 × MIR604 × NK603, known by its trade name Optimum™ Intrasect Xtreme, which was obtained by the crossing among five individual events. In the same way that occurs with the re-transformation process, in the sexual cross between transgenic events, the genes will be introduced in different positions of the genome, resulting in segregation in the next generations. In this way, it is necessary to maintain a large number of the progeny for evaluation, demanding cost, time and labor (QUE et al., 2010).

#### *Plant Minichromosomes for genetic engineering as a future potential for stacking genes*

Minichromosomes or Plant Artificial Chromosomes (PAC) platforms have been developed as super vectors for foreign gene organization, expression and manipulation for genetic engineering use (YU et al., 2007). A minichromosome is an extremely small version of a chromosome, including the basic components necessary for replication (centromere, telomeres, and replication sequences). They are stable and replicate autonomously in the cell during cellular division to allow the resident genes to be faithfully expressed and transmitted from cell to cell and from generation to generation (YU et al., 2016). To allow the manipulation of genes, minichromosomes are usually designed to have site-specific recombination (SSR) systems to allow addition, deletion and replacement of genes or direct editing with recent genome-editing technology (YU et al., 2016). The minichromosomes have been constructed in important plant species including maize (YU et al., 2007), rice (XU et al., 2012) and barley (KAPUSI et al., 2012).

Plant minichromosomes have the potential for stacking multiple traits on a separate entity from the native genome. Introducing transgenes into

minichromosomes does not have the risk of insertion causing disrupting of native genome elements and transgenes on minichromosomes can be transferred between lines without the linkage to any native genes (GRAHAM et al., 2015).

The microchromosome strategy is expected to overcome the limitations on delivery of large DNA sequences, including large genes, multigene complexes, or even complete and multiple metabolic pathways, since it has the potential to transfer tens or hundreds of transgene simultaneously (NAQVI et al., 2010). However, no commercial product has yet reached the market using this technology. This may be related to the technical difficulties associated with the assembling of artificial chromosome, stability in the transmission to the next generation, as well regulatory issues.

The gradual reduction in the cost of deregulation of GMOs may facilitate the application of PAC technology in plant breeding (YU et al., 2016). Also, newly emerging synthetic biology methods used for producing and transplanting whole bacterial genomes may simplify the process, and reduce the cost of minichromosome implementation (GLASS et al., 2018). Improved PAC technology should play an important role in genetic engineering to produce more and higher quality agricultural and industrial products to meet future demands (YU et al., 2016).

#### *Commercially released stacked crops and characteristics*

According to the GM Approval database of the International Service for the Acquisition of Agri-Biotech Applications (ISAAA), publicly available at <http://www.isaaa.org/gmapprovaldatabase/>, thirteen crops with gene stacking, belonging to 341 events, were approved until 2019 and they are presented in table 1. The genes and traits were pooled together in this table for ease of presentation. However, the ISAAA GM database offers details on individual events, including the developer company, method of gene insertion, countries in which a particular event has been authorized, as well as regulatory information.

Maize is the crop with the largest number of event with gene stacking, followed by cotton, potato, canola and soybean. In these crops, the main traits are herbicide tolerance and insect resistance. However, other characteristics of interest include drought tolerance, production of long chain Omega-3, a pollination control system, resistance to viral diseases, and change in product quality and alteration in the content of oils and fatty acids (Table 1).

A smaller number of stacked events are approved for alfalfa, rice, carnation, chicory, poplar, rose, beet and squash. In glyphosate herbicide tolerance alfalfa, low lignin content was obtained through gene silencing by the introduction of the inverted (antisense) gene sequence of the *Caffeoyl-CoA O-methyltransferase (ccomt)*, a key enzyme involved in lignin production. In this case, the inserted gene produces an antisense RNA, complementary to the sense target mRNA. The hybridization of the sense and antisense RNA strands results in the degradation of the *ccomt* mRNA, preventing its translation. In carnation and rose, the color modification was executed by the insertion of genes involved in anthocyanins production, resulting in the alteration of the color and quality of the commercial product (Table 1).

In rice, besides the stacking of *cry* genes conferring resistance to Lepidoptera, the golden rice containing the insertion of two genes, phytoene desaturase (*crt1*) and phytoene synthase (*psy1*) resulting in greater content of provitamin A, was recently released commercially in Australia (2017), New Zealand (2017), Canada (2018) and USA (2018) (Table 1).

The DHA Canola event was created by stacking seven genes: *Lackl-Delta12D*, *PICPA-Omega-3D*, *Micpu-delta-6D*, *Pyrco-delta-6E*, *PAVSA-Delta-5D*, *Pyrco-delta-5E* and *PAVSA-delta-4D*. These genes are involved in the metabolic pathway leading to long chain Omega-3 production and this stacking event increase production of this nutritionally desirable fatty acid. The discovery, introduction and coordinated expression of transgenes coding for a complete biosynthetic pathway with enzymatic conversions for the production of Omega-3 was one of the most complex introductions achieved in plant metabolic engineering (SINGH, 2010).

The potato event X17, registered under the trade name Innate® Aclimate, was recently released in USA (2016), Australia, Canada and New Zealand (2017). This event has six stacked genes to confer resistance to potato late blight, caused by *Phytophthora infestans*, and modified product quality. For the development of this product, the RNAi gene silencing technology was used to regulate the expression of genes responsible for the enzymatic browning process. The introduction of a gene encoding a RNA that contains small sense and antisense sequences of the gene of the interest, produces a RNA hairpin that triggers the degradation of target homologous mRNA by a silencing system involving a very specific group of plant enzymes (RISC and DICER), resulting in



Table 1 - List of crops globally released commercially with gene stacking, number of events, genes inserted and traits conferred by the genes (Source: ISAAA, 2019).

Crop	Number of events	Genes involved in stacked events*	Characteristics determined by the stacked genes
Alfalfa	1	<i>cp4 epsps (aroA:CP4); ccomt</i> (inverted repeated)	Glyphosate herbicide tolerance and lignin reduction
Canola	25	<i>bar; barnase; barstar; cp4 epsps (aroA:CP4); gat4601; goxv247; pat (syn), Lackl-delta12D, Picpa-omega-3D, Micpu-delta-6D, Pyrco-delta-6E, Pavsa-delta-5D, Pyrco-delta-5E, Pavsa-delta-4D</i>	Glyphosate and glufosinate-ammonium herbicide tolerance, pollination control system and increased nutritional quality (long-chain omega-3 production)
Carnation	4	<i>bp40 (β3'5'h); cytb5; dfr; dfr-diac; sfl (β3'5'h); surB</i>	Sulfonylurea herbicide tolerance and alteration of coloring
Chicory	3	<i>bar; barnase</i>	Glufosinate-ammonium herbicide tolerance and pollination control system
Cotton	39	<i>2mepsps; aad-12; bar; bxn; cp4 epsps (aroA:CP4); cpTI; CryIA; cry1Ab; cry1Ac; cry1F; cry2Ab2; cry2Ae; dmo; pat; vip3A(a)</i>	Glyphosate, oxynil, glufosinate-ammonium, 2,4-D and dicamba herbicide tolerance; lepidopteran and coleopteran insect resistance
Maize	206	<i>2mepsps; aad-1; amy797E; bar; cordapA; cp4 epsps (aroA:CP4); cry1A; cry1A.105; cry1Ab; cry1Ab (truncated); cry1Ac; cry1F; cry1Fa2; cry2Ab2; cry2Ae; cry34Ab1; cry35Ab1; cry3Bb1; cry9C; cspB; dam; dmo; dvsnf7; ecry3.1Ab; gat4621; mcry3A; mepsps; moery1F; pat; pat (syn); pinII; vip3A(a); vip3Aa20; zm-hra</i>	Glyphosate, 2,4-D, glufosinate-ammonium, dicamba and sulfonylurea herbicide tolerance, coleopteran and lepidopteran insect resistance, nutritional quality (lysine increase, increased ethanol production by amylase thermoestability), drought tolerance and pollination control system
Poplar	1	<i>cry1Ac; aPI</i>	Lepidopteran insect resistance
Potato	29	<i>asn1; cp4 epsps (aroA:CP4); cry3A; plrv_orf1; plrv_orf2; ppo5; pvy_cp; rpi-vnt1</i>	Coleopteran insect resistance, viral diseases resistance, glyphosate herbicide tolerance and quality (reduction of asparagine and darkening)
Soybean	25	<i>2mepsps; aad-12; avhppd-03; cp4 epsps (aroA:CP4); cry1A.105; cry1Ac; cry1F; cry2Ab2; dmo; fad2-1A (sense and antisense); gat4601; gm-fad2-1 (partial sequence); hppdPF W336; Nc.Fad3; pat; pj.D6D</i>	Glyphosate, 2,4-D, mesotrione, dicamba, isoxaflutole and glufosinate-ammonium herbicide tolerance, lepidopteran insect resistance, quality (fatty acids and oil) and drought tolerance
Squash	2	<i>cmv_cp; wmv_cp; zymv_cp</i>	Resistance to viral diseases
Sugar Beet	1	<i>cp4 epsps (aroA:CP4), goxv247</i>	Glyphosate herbicide tolerance
Rose	2	<i>bp40 (β3'5'h), 5AT</i>	Changing the coloring
Rice	3	<i>Cry1Ab, Cry1Ac, bar, crt1, psyl</i>	Lepidopteran insect resistance, glufosinate-ammonium herbicide tolerance and increased nutritional quality

\*Genes involved in different events are pooled together. The marker genes were not taking in to account to prepare this list, unless it confers important trait for the crop being considered.

the silencing of the target gene. As a result, Innate potatoes are less susceptible darkening and the onset of black spot from bruising caused by impact and pressure during harvest, storage and food preparation. Also Innate potatoes have lower levels of asparagine and reducing sugars, which decreases the potential formation of acrylamide.

Pathogenic virus resistance has also been a target of gene stacking strategies, especially in potato and squash. The potato event RBMT15-101, trade name: New Leaf<sup>™</sup> Y Russet Burbank potato, carries the *Cry3A* gene for Coleopteran insect resistance

and the coat protein of the potato virus Y (PVY) that confers resistance to potato virus Y (PVY). Transgenic squash was engineered for resistance to 3 different virus (Cucumber Mosaic Cucumovirus, (CMV), Zucchini Yellow Mosaic Potyvirus (ZYMV) and Watermelon Mosaic Potyvirus 2 (WMV2), mediate by the expression of virus coat protein (Table 1).

Herbicide tolerant plants were the first available in the market and remain the most cultivated (ISAAA, 2018). Tolerance to herbicide glyphosate is one of the most commonly engineered traits and is mainly conferred by versions of the

*epsps* gene that encodes versions of the enzyme 5-enolpyruvylshikimate-3-phosphate synthase that make the plants insensitive to glyphosate (ISAAA, 2019). The occurrence of glyphosate resistant weeds led to the search for other genes. It was verified that the stacking of *epsps* and *gat* genes (*gat* codes for a glyphosate N-acetyltransferase enzyme that acts as an alternate method of glyphosate degradation), increases the tolerance level and reduce the accumulation of glyphosate in plants (LIANG et al., 2017). Other engineered strategies of herbicide tolerance commercially available include cultivars with genes conferring alternatives herbicide tolerance such as the events: CV127, trade name Cultivance, with the *acetohydroxyacid synthase (csrl-2)* gene conferring sulfonylurea herbicide tolerance; DAS68416-4, trade name Enlist™ Soybean, with the *pat* gene conferring glufosinate-ammonium herbicide tolerance; MON-87708-9, trade name Genuity® Roundup Ready™ 2 Xtend™, with the *dicamba mono-oxygenase (dmo)* gene conferring dicamba herbicide tolerance, and, MON87427 x MON89034 x TC1507 x MON87411 x 59122 x DAS40278, trade name SmartStax™ Pro x Enlist™, with *aad-1* gene conferring 2,4-D herbicide tolerance (ISAAA, 2019).

The second largest group of single gene transgenic plants cultivated worldwide was those engineered for insect-resistant. Most of the approved events that present resistance to insects were obtained by the insertion of *Cry* genes originating from the entomopathogenic bacterium *Bacillus thuringiensis*. These plants are commonly known as Bt plants. The first generation of Bt plants expressed only one toxin. However, due to the emergence of cases of resistance and due to demand for plants resistant to a wider range of insects, the second-generation plants feature gene stacking. Those expressing more than one CRY protein targeting the same species of insect are important tools to delay the emergence of resistance to Bt technology, following the multiple attack strategy (CARRIÈRE et al., 2015).

Although, early commercial adoption of genetically modified plants was done so with predominantly with herbicide tolerance and insect resistance conferred by Bt genes; one of the great demands for modern agriculture is production under unfavorable climatic condition. The plant tolerance response to abiotic stresses is extremely complex and involves several biochemical and physiological processes. Thus, the improvement in tolerance to abiotic stresses possibly will be directly linked to gene stacking, especially when one wishes to combine tolerance to multiple abiotic stresses such as drought and salinity (SHEN et al., 2015).

Transgenic cotton plants, expressing the *Arabidopsis vacuolar Na<sup>+</sup>/H<sup>+</sup> antiporter (AtNHX1)* gene and *H<sup>+</sup>-pyrophosphatase (AVP1)* gene, produced by cross-pollination of two single-gene-overexpressing plants produced significantly more biomass and the highest yields than all other plants under salt and drought stress conditions. The authors verified that the co-overexpression of AVP1 and AtNHX1 leads to higher drought and salt tolerance than AVP1 or AtNHX1 single-gene overexpression in transgenic plants (SHEN et al., 2015).

### Regulation

The data necessary for the regulation of a new transgenic event include: (1) molecular characterization of the gene insertion, including the flanking sequences of the transgene in the host genome and the correct position of the promoters and terminators; (2) similarity assessment of sequences inserted with known allergens and toxins sequences through bioinformatics analysis; (3) studies of protein toxicity; (4) analysis of the physico-chemical properties of the proteins inserted as well as their stability and activity; (5) protein quantity expressed in plant; (6) analysis of nutritional composition and animal feed studies for the evaluation of nutritional equivalence; and, (7) assessment of human food exposure. Each regulatory agency may have some specific requirements, but in general, this data meets most of the requirements of the regulatory agencies of countries that plant and import transgenic products (KRAMER et al., 2016). These demands fall into the requirements for the regulation and release of new transgenic events, whether they are produced as single gene or with genetic stacking.

Most of the events approved with stacked genes were obtained by sexual crossing between transgenic events. In this methodology, the transgene(s) inserted in each individual event are transferred from the parent to the progeny in a similar way to that occurring with endogenous genes. There is no new recombination of DNA as can occur in a process of genetic transformation by bombardment or *A. tumefaciens* (PILACINSKI et al., 2011).

The crossing between events that have not been evaluated and approved must follow the same standards of evaluation and regulation of a new event. However, this is a great deal of controversy over how to regulate transgenic events obtained by crossing between already evaluated and approved events. There is no consensus among regulatory agencies as to which evaluations are necessary to prove the safety of these new plants with genes combination.



In countries of the European Union, a transgenic plant obtained by crossing between two approved transgenic events is considered as a new event and is subject to the same regulatory requirements as the original events (De SCHRIJVER et al., 2007; PILACINSKI et al., 2011).

Conversely, some regulatory agencies such as in the United States, Canada and Australia follow the precept that if parental were evaluated and considered safe, the resulting progeny will also be, and thus not requiring additional regulatory data. This is provided that the genes and proteins expressed are not subject to interaction that could affect the safety of these products. Therefore, in many cases, the safety assessment of the simple events can be applied the plants resulting from the combination of events (PILACINSKI et al., 2011).

Other countries require variable amounts of data to confirm that the security assessments of simple events are valid for the combination of events, and it is not necessary to develop the entire regulatory process. In Brazil, plants resulting from the combination of events by the conventional crossing are considered as a new event, but it is not necessary to perform the entire risk assessment process if the parent has already been evaluated and approved, making the regulatory process simpler and faster (CTNBIO, 2009; AGAPITO-TENFEN et al., 2014).

The main concerns regarding the cross between transgenic events are: (1) the stability of the inserts; (2) transgene expression; and (3) potential synergic or antagonistic effects between genes and proteins (KOK et al., 2014). Regarding the insertion stability, DE SCHRIJVER et al. (2007) suggested the need for genotypic data obtained by molecular techniques to demonstrate that there was no alteration in the molecular structure of the gene during the insertion process. The DNA sequencing and bioinformatic analyses must ensure the absence of specific mutations, rearrangements in the sequences of inserts and flanking regions. Conversely, there is data that proved that transgenes, after entering the genome, behave like endogenous gene and follow the laws of Mendelian inheritance. The security of the combination of transgenic events is based on the principle that plant breeding is a safe process. In this way, the crossing between two transgenic events does not present greater risks than the cross between a transgenic event and a non-GMO genotype, or between two conventional genotypes in relation to gene stability (PILACINSKI, et al., 2011; KOK et al., 2014).

In relation to the transgene's expression, the insertion of a gene into a new genetic background

can alter the gene's expression, as can other factors, such as the environment and the intrinsic variation of the culture (KOK et al., 2014). However, biological relevance should be considered in relation to the effectiveness of the expressed proteins.

In the evaluation of maize resulting from the crossing between the events MON-89 Ø 34-3 (genes *cry1A.105* and *cry2AB2*) X MON-ØØ6Ø 3-6 (gene *epsps*), it was determined that the stacking events resulted in a reduction of the transgenes mRNA. However, the authors did not evaluate the protein content in the plants and the possible interference in the effectiveness of insect control and tolerance to glyphosate herbicide (AGAPITO-TENFEN et al., 2014). The concentration of BT protein and the potential to insect pest control of the stacking events is important because the exposure of target insects to decreased Bt expression is a concern for insect resistance management (MARQUARDT et al., 2013).

In relation to the potential synergic or antagonistic effects among genes and proteins, it is not expected that there will be interactions in plants when: (a) genes do not share the same intermediate metabolites and are not on the same metabolic route, and/or; (b) have different mechanisms of action that are distinct at the biochemical or cellular level (PILACINSKI et al., 2011). In this way, the understanding of the expression profile and the mechanisms of action of individual transgenes can be used in a case-by-case approach to identify potential interactions among transgenes or their products. Therefore, when there is some possibility of interaction, the appropriate measures may be taken to confirm or update the biosecurity information available (KRAMER et al., 2016).

Due to the discussion of the safety of hybrids resulting from cross-genetic events, some studies have been developed to evaluate the equivalence, stability and safety of these hybrids. Nutritional equivalence and phenotypic characteristics were demonstrated between the maize hybrid TC1507 (genes *cry1F* and *pat*) X DAS-59122-7 (genes *cry34AB1*, *cry35AB1* and *pat*) and the conventional isogenic hybrid (FERREIRA et al., 2015). The maize hybrid MON 89034 (genes *cry2AB2* and *cry1A. 105*) x TC1507 (genes *cry1FA2* and *pat*) x MON 88017 (genes *cry3BB1* and *cp4 epsps*) x DAS-59122-7 (genes *pat*, *cry34AB1* and *cry35AB1*) produce eight proteins for herbicide tolerance, Coleopteran and Lepidopteran resistance. When assessed the composition, the hybrid was comparable to conventional control in nutrient levels, antinutrients, secondary metabolites in fodder

and grain (LUNDRY et al., 2013). The data obtained after 15 years of studies with six maize hybrids resulting from different combinations between the BT11 events (*pat* and *cry1AB* genes), GA21 (gene *mepsps*), MIR604 (gene *mcry3A*) and MIR162 (gene *vip3AA20*) did not demonstrate evidence of substantial changes in composition, expression or stability of the insert due to the additive effects synergists of the combination of events by sexual crossing (KRAMER et al., 2016).

## FINAL CONSIDERATIONS AND CONCLUSION

Considering the advances in the areas of sequencing and bioinformatics allied to the techniques of molecular biology and biotechnology, we should witness an increase in the number of genes sequenced and identified with potential for use in genetic transformation suitable for use in increasing plant health, quality, productivity and production in abiotic stress conditions. The tendency is that gene stacking will increase as a mechanism for the production of commercial engineered varieties of all crop types, with an increase in the number of genes and characteristics combined in the same plant.

New methods will be used together with genetic engineering allowing greater control over the process of introducing genes into plants, including the insertion site, expression profile and segregation of transgenes. The use of genome recombination and editing systems will allow the insertion or withdrawal of genes in specific locations of the plant genome. The CRISPR/Cas9 gene-editing system, for example, is able to generate heritable, targeted mutations, resulting in a non-transgenic mutant plant. Also, it can be used to add a gene in a specific place in genome, since the system carries a guide RNA presenting homology to the target sequence position of the genome.

The development of artificial chromosomes of plants creates the possibility of building super vectors for the organization, expression and manipulation of tens and hundreds of exogenous genes for the next generation of genetic engineering. Looking ahead, the development of the synthetic biology and synthetic genomes, which allow to engineering and manipulation of an organism's genetic material on the scale of the whole genome, will allow the construction and transformation of crops with multiple genes, traits and functions to produce more agricultural products to meet future demands. Transgenic technology will continue to

have a significant influence on the development of our society, mainly by providing incremental improvements in crop production, nutritional value and crop protection in the face of growing food demand and climate changes.

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## DECLARATION OF CONFLICT OF INTERESTS

The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

## AUTHORS' CONTRIBUTIONS

The authors contributed equally to the manuscript.

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