Editor's Choice: Crop Genome Plasticity and Its Relevance to Food and Feed Safety of Genetically Engineered Breeding Stacks[1]

Genetically engineered (GE) stacks, combinations of two or more single transgenic events (i.e. single-locus insertions) that have been produced by crossing sexually compatible parents, are an important and growing sector of the crop seed market. Stacked traits covered 26% of the global transgenic crop area in 2011 and were the fastest growing trait group, with a 31% increase in the area planted compared with 2010 (James, 2011). Stacked traits already dominate the market in some regions. For example, 95% of the cotton (*Gossypium* spp.) grown in Australia during 2011 had both herbicide tolerance and insect resistance traits (James, 2011). Worldwide, at least 12 countries are now growing stacked varieties, of which nine are developing countries (James, 2011). The rapid adoption of GE stacks has focused attention on whether the safety of such products differs from that of the individual events.

At issue is whether combining two or more events via conventional breeding creates changes that require additional safety assessment, even though the safety of each event in the stack has been assessed previously. The two main concerns are (1) whether combining two or more events into a plant by conventional breeding increases genomic instability and (2) whether potential interactions between the products of the transgenes in GE stacks impact safety. This paper draws on insights from plant breeding, describes the plasticity of conventional plant genomes over generations of crossing and selection, and considers the implications of event stacking on food and feed safety in the context of the normal plant genome.

The term GE is used here to distinguish the process of specific, intentional, and directed physical modification of the genome of a plant from random genetic modifications that occur in conventional breeding or by mutagenesis. The term GE is preferred over the term "genetically modified" (commonly referred to as GM) for these reasons. The term "event" refers to a single-locus insertion of recombinant DNA into the host genome regardless of the number of genes contained on the inserted piece of DNA. The term "conventional breeding" refers to methods of crossing plants with desired characteristics to generate offspring combining those desirable characteristics. These characteristics may include both non-GE and GE traits.

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SCOPE

This paper focuses on (1) the potential of transgenes to alter genome stability and (2) the potential risks to food and feed safety associated with genome instability. A companion paper focuses on potential interactions that can occur from transgene stacking (H.Y. Steiner, C. Halpin, J.M. Jez, J. Kough, W. Parrott, L. Underhill, N. Weber, and L.C. Hannah, unpublished data). The potential environmental impact that may arise from the cultivation of crops with GE stacks is outside the scope of these articles. Crops containing a single event, that carry multiple traits that are introduced simultaneously (i.e. molecular stacks resulting from cotransformation), or that are produced by retransformation of an event require a de novo safety assessment, as is customary for all new events, and, hence, are also outside the scope of this paper.

STACKING OF ENDOGENOUS GENES IS COMMON IN PLANT BREEDING PROGRAMS

Plant breeding is a major underpinning of modern agriculture, as it creates varieties containing multiple desirable traits through the stacking of both known and many unknown genes. While increasing yield potential is a major objective, protecting yield potential (i.e. breeding for resistance to biotic and abiotic stresses) is also critical. Many stress resistance genes have come from related species such as wild relatives of crop plants. Hajjar and Hodgkin (2007) reported that conventional breeding efforts in 19 of the world's major crops had incorporated 111 genes from wild relatives into new varieties over the previous 20 years. Eighty percent of these genes confer disease resistance; the remainder control abiotic stress resistance or quality traits (Hajjar and Hodgkin, 2007).

Modern non-GE crop varieties differ mainly from their predecessors by the incorporation and stacking of genes from distant relatives. For example, IR8 rice (*Oryza sativa*), released in 1966, is resistant to the green leafhopper (*Nephotettix* spp.) and moderately resistant to salinity, rice blast, and phosphorus deficiency. Just 11 years later, IR42 was released, which possessed resistance to multiple diseases (rice blast, *Grassy stunt virus*, *Rice tungro virus*, *Ragged stunt virus*, and bacterial blight), pests (brown planthopper [*Nilaparvata lugens*], stem borer), and abiotic stresses (nitrogen deficiency, zinc deficiency, iron deficiency, alkalinity, and iron toxicity; International Rice Research Institute, 1981). Today, many seed catalogs list the multiple resistance traits present in each variety. Although the genetic and biochemical bases for these traits

are usually not well understood, conventional breeding has successfully stacked traits to create stable crop varieties that are considered safe and that, in most cases, were not subjected to safety assessments prior to commercialization.

Gene introgression from one species into another via sexual cross-breeding is inherently imprecise: additional genes contained in the DNA linked to the desired gene are also incorporated. For example, transfer of the *Tm-2* gene from *Solanum peruvianum* to tomato (*Solanum lycopersicum*) provides resistance to *Tobacco mosaic virus*. Four to 51 centimorgans of *S. peruvianum* DNA accompanied the *Tm-2* gene when introgressed into different tomato varieties (Young and Tanksley, 1989). One centimorgan of DNA can contain up to several hundred thousand bases of DNA sequence and include multiple genes. Similarly, modern wheat (*Triticum aestivum*) varieties contain resistance genes from dozens of species representing six related genera. In some cases, entire chromosomes or chromosome arms representing millions of base pairs of DNA were transferred along with the desired resistance gene (Jones et al., 1995). Despite this imprecision, plant breeding has a remarkable record of safety; no newly released variety has had any novel or previously unknown food or feed hazard (H.Y. Steiner, C. Halpin, J.M. Jez, J. Kough, W. Parrott, L. Underhill, N. Weber, and L.C. Hannah, unpublished data).

WHAT MECHANISMS ALTER GENOMIC STABILITY IN PLANTS?

The primary way through which the presence of two or more transgenes could give rise to genetic instability in a GE stack, but not in the two transgenic parents, is through homologous recombination between sequences repeated in the transgenes. Therefore, it is necessary to first evaluate the natural stability of non-GE plant genomes and the role played by repeated sequences in creating genomic changes. Genomic diversity is caused by plasticity: the capacity of the genome to reorganize itself (Nevo, 2005). These changes involve alterations in both sequence (McClintock, 1984) and chromatin state (Brink, 1960; Hale et al., 2007), providing genetic variation for selection by breeders (Vyskot et al., 1991; Morgante et al., 2007). A large measure of the plasticity is the direct result of genome organization at the DNA level (Fig. 1). As discussed in the following sections, plant genomes contain substantial numbers of mobile (transposable) DNA elements and other repetitive coding and noncoding DNA sequences, all of which lead to genome diversity.

Repetitive DNA Sequences Are Major Components of Plant Genomes and Contribute to Genomic Plasticity

Even coding genes are seldom unique in a plant. A myriad of biochemical, physiological, and genomic investigations definitively establish that a high percentage of genes are present in duplicated forms or as

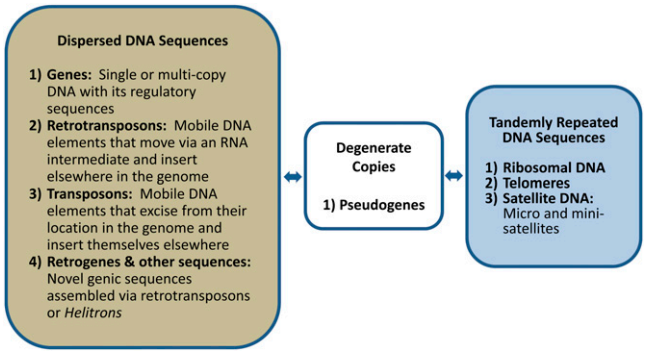

Figure 1. Overview of the types of DNA in plants and their genomic arrangement. DNA sequences present in multiple copies, including mobile elements such as DNA transposons and retrotransposons, are important contributors to genomic plasticity.

members of multigene families. Multigene families have arisen through duplication of whole genomes, as seen in rapeseed (*Brassica napus*), or of smaller genomic regions (Moore and Purugganan, 2005; Cheung et al., 2009).

Other types of repetitive DNA include ribosomal genes and telomeres, both of which consist of tandem repeats. Tandem DNA repeats are particularly prone to changes in copy number resulting from unequal crossing over, gene conversion, intrastrand recombination, or replication slippage (Flavell, 1985; Strand et al., 1993). For example, such mechanisms generate new alleles of disease resistance genes (Kuang et al., 2004), which are commonly found in groups of related sequences called resistance gene clusters.

Short stretches of repeated DNA sequences, called microsatellites, simple tandem repeats, or simple sequence repeats, are another type of repetitive DNA with a tandem arrangement. The mutation rate for copy number of dinucleotide repeats in maize (*Zea mays*) is almost eight for every 10,000 meioses (Vigouroux et al., 2002), or one change in every 625 seeds, while the calculated mutation rate for microsatellites in durum wheat (*Triticum turgidum*) is 2.4 per 10,000 meioses (Thuillet et al., 2002).

Any closely linked duplicated DNA sequences have the potential to undergo homologous recombination, leading to the excision of the intervening DNA element. For example, many retrotransposons have long terminal repeat sequences (LTRs). The barley (*Hordeum vulgare*) genome contains many LTRs that lack intervening sequences, suggesting that pairing of long terminal repeats and subsequent homologous recombination deleted the intervening sequence (Shirasu et al., 2000). Moreover, the excised intervening DNA can replicate as extrachromosomal circular DNA and insert into other regions of the genome (Cohen et al., 2008; Navrátilová et al., 2008).

In some cases, excision of the DNA between two linked copies of a gene in inverse orientation results in gene silencing (Kusaba et al., 2003); the presumed mechanism is that once the DNA between the two genes is removed, read-through transcription of the

antisense strand leads to double-stranded RNA, triggering silencing. One example of such excision-induced silencing was found at the rice glutelin locus (Kusaba et al., 2003).

Recombination between duplicated genes can create novel alleles. One example is the *anthocyaninless1* (*a1*) locus of maize, which controls seed color (Yandeau-Nelson et al., 2006). Another is the *I* (for inhibitor of color) locus in soybean (*Glycine max*), which consists of both tandem and inverted clusters of genes coding for chalcone synthase. Spontaneous reversion from buff to black seed coat color is associated with deletion of a whole cluster within the *I* locus (Tuteja et al., 2004), presumably through homologous recombination.

The vast majority of homologous recombination that causes heritable changes occurs at meiosis. Homologous recombination is controlled by highly conserved meiotic pairing machinery that leads to strict pairing of homologous sequences between homologous chromosomes or sister chromatids (for review, see Hamant et al., 2006). Homologous recombination also can occur in somatic cells, with recombination rates between homologous alleles ranging from 5.74×10^{-5} cells in soybean to 7.7×10^{-6} cells in tobacco (*Nicotiana tabacum*; Evans and Paddock, 1976). In Arabidopsis (*Arabidopsis thaliana*), the frequency of homologous recombination in somatic tissues is between 10^{-6} and 10^{-7} per genome (Swoboda et al., 1994). In contrast, meiotic recombination rates are no less than one recombination for every chromosome pair, and the rate of recombination between alleles of one gene is usually about one exchange per 1,000 meiotic events.

Recombination between homologous sequences located in nonallelic positions within a genome is rare. However, such recombination of homologous sequences on nonhomologous chromosomes may be an underlying cause of both inversions and translocations (Fig. 2; for review, see Gaut et al., 2007). Large-scale chromosomal changes such as inversions or translocations result in decreased fertility and, in some cases, deviations from Mendelian segregation ratios (Burnham, 1962; Singh, 2003).

Nonhomologous Recombination and Double-Strand Breaks Contribute to Genetic Change

Double-strand breaks are common in plants (Gorbunova and Levy, 1997, 1999). They are necessary for meiotic recombination (Gerton and Hawley, 2005) and can be caused by transposon excision or integration of DNA sequences. Following a double-strand break, DNA-repair mechanisms use other DNA strands as templates for DNA synthesis for break repair (Puchta, 2005; Wicker et al., 2011). For example, from 1 to 131 bp of filler DNA was added during the repair of spontaneous 60- to 880-bp deletions in the *waxy* locus of maize (Wessler et al., 1990). Double-strand breaks can lead to the rearrangement of DNA by recombination between homologous but nonallelic sequences (ectopic recombination) at a very low frequency (Shalev and Levy, 1997; Puchta, 1999). They also copy gene-containing DNA sequences up to 20 kb in length to new locations when the template used for repair comes from a nonhomologous chromosome in the vicinity (Wicker et al., 2010). Ultimately, such double-strand DNA break repairs, with their associated deletions and additions of DNA, may even contribute to changes in plant genome size (Kirik et al., 2000; Puchta, 2005).

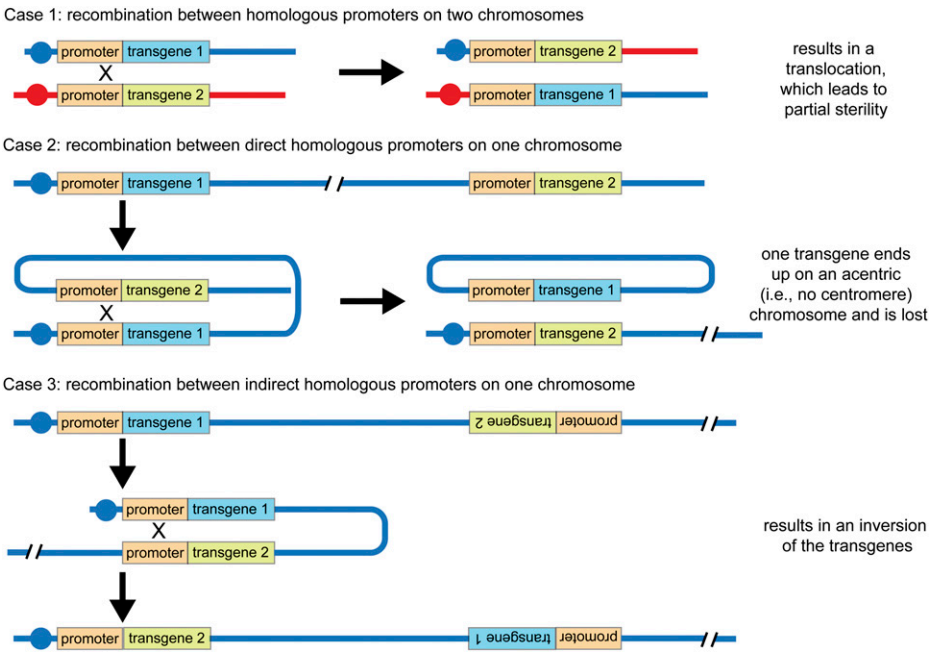

Figure 2. Recombination between homologous DNA sequences has been proposed as a cause of both translocations and inversions. Three cases (recombination between homologous promoters on different chromosomes, between homologous promoters in direct orientation on one chromosome, and between homologous promoters in indirect orientation on one chromosome) are shown, and their results are indicated.

CAN GENOME INSTABILITY COMPROMISE FOOD/FEED SAFETY?

Although the presence of a few additional duplicated sequences derived from transgenes is unlikely to result in a measureable increase in genome instability, it is still pertinent to address the ability of such changes to compromise food and feed safety, since it has been argued that plants contain dormant metabolic changes that could become active due to genomic instability of various types (Kessler et al., 1992).

In addition, Latham et al. (2006) and Wilson et al. (2006) reported that the transformation process itself is mutagenic. Their assertion is based on analyses of Arabidopsis plants engineered without the use of tissue culture that show mutations not associated with the integration of the transgene. It is not possible with the currently available data to determine if these mutations are more frequent than the normal background mutation rate measured by Ossowski et al. (2010). Regardless, their concerns about mutations produced by the transformation process are not relevant to GE stacks, as any unintended effects would be evaluated during the safety assessment of the individual events.

In order to evaluate the impact of mutations and other types of instability on safety, it is first necessary to review additional types of mutations and other genomic changes that can happen naturally in plant genomes.

Transposable Elements Play a Dominant Role in Altering Genomes

Genomic change can occur through insertion or excision of transposable elements (Wessler, 2006). Mobile DNA elements represent 50% to 80% of the genome in species such as barley and maize (Feschotte et al., 2002). The sequenced cacao (*Theobroma cacao*) genome has 28,798 predicted protein-coding genes and 67,575 transposable elements (Argout et al., 2011). Some elements insert at random, some insert near genes, and some insert preferentially into centromeric or heterochromatic areas (Schnable et al., 2009). Transposition of mobile elements can be activated by mutagenesis (Anderson, 1948; McClintock, 1950; Gowda et al., 2011), transformation (Wu et al., 2009), tissue culture (Peschke et al., 1987; Hirochika et al., 1996; Jiang et al., 2003; Barret et al., 2006; Gowda et al., 2011; Smith et al., 2012), wide hybridization (Liu and Wendel, 2000; Wang et al., 2010; Zou et al., 2011), and stress (Lin et al., 2006; Gowda et al., 2011).

Transposable elements fall into two general types: retrotransposons and DNA transposons. Retrotransposons move when the DNA encoding the element is transcribed into RNA that is then reverse transcribed back to DNA prior to insertion into the genome (Wang et al., 2006). DNA transposons consist of DNA that can physically excise from the chromosome and integrate elsewhere in the genome. Some classes of transposons in maize are associated with the natural induction of large-scale chromosomal deletions, duplications, and inversions (Zhang and Peterson, 1999; Zhang et al., 2006). Most often, however, their insertion in or near a gene can disrupt it, and subsequent excision can restore gene function. Such reversion phenotypes can sometimes be seen by eye and were instrumental in the discovery of transposable elements (McClintock, 1948). Certain phenotypes in edible crops are caused by a mutation from transposable elements. For example, the wrinkled pea (*Pisum sativum*) mutation studied by Mendel is the result of a transposon insertion into the gene responsible for starch branching (for review, see Vollmann and Ruckenbauer, 1997). Frequently, transposon excision is imprecise and results in an altered DNA sequence, or "footprint." Such changes in nucleotide sequences can also alter splicing of RNA transcripts (Giroux et al., 1994; Feschotte, 2008), thereby creating novel proteins. These footprints occasionally result in the addition of amino acids to a wild-type protein sequence. For example, two amino acids were added from *Dissociation* element excision to the large subunit of adenosine diphosphoglucose pyrophosphorylase, leading to an increase in seed weight (Giroux et al., 1996). The vast majority of changes, however, can be expected to be either neutral or negative in terms of plant fitness and human preferences for cultivation or consumption.

Helitrons are a recently described class of eukaryotic transposon that likely underlie many of the genotype-specific differences in the dispensable genome (Lal et al., 2003, 2009; Gupta et al., 2005; Morgante et al., 2005). *Helitrons* capture pieces of genes from throughout the genome and assemble them into novel combinations within the element. Expression of these chimeric coding regions can give rise to the synthesis of novel proteins. A comparison of maize inbreds B73 and Mo17, two lines of historical importance in maize breeding programs, revealed approximately 10,000 gene fragments found only in one inbred but not the other and that might have been mobilized by *Helitrons* (Lal et al., 2009). Judging from the characterization of randomly chosen *Helitrons*, Barbaglia et al. (2012) provide a minimum estimate of 11,000 unique transcripts arising from expressed chimeric genes assembled by *Helitrons*.

Other transposable elements with the ability to capture exons or entire genes include the Pack-MULE (Jiang et al., 2004; Hanada et al., 2009) and *Anaconda* (Ohtsu et al., 2005) elements in rice. Of 898 such transposon-derived DNA fragments identified in rice, 55% seem to be expressed, with approximately 35% being chimeric in nature. Based on the synonymous substitution rate, some of these fragments have appeared more recently than others, suggesting that these genes are created at a slow but steady rate. Those that do not produce functional proteins are called pseudogenes (Wang et al., 2006).

The *wp* flower color mutation in soybean is one example of such transposon-mediated gene capture. This mutant has a very pale pink flower color, and its seeds have 4% more protein and are 22% larger than those of its progenitor. The change is due to a 5.7-kb insertion of the *Tgm-Express1* transposon into the flavanone 3-hydroxylase 1 gene, which conditions purple flower color. The transposon itself contains partial copies of five genes involved in amino acid synthesis or sugar

metabolism (Zabala and Vodkin, 2005) that are properly recognized as exons (Zabala and Vodkin, 2007), suggesting that the transposon might function as a new gene. In maize, a newly discovered chimeric gene expressed in early ear development appears to have been formed by retrotransposon-mediated shuffling between three genes (Elrouby and Bureau, 2010). In cultivated tomato, retrotransposons altered gene expression by linking exons from the β-subunit of inorganic pyrophosphate-dependent phosphofructokinase to those of the homeobox gene *LeT6* (for *Lycopersicon esculentum* T6), leading to the mouse-ear phenotype (Chen et al., 1997). In another example, the elongated fruit of some tomato varieties is due to the retrotransposon-mediated duplication of a 24.7-kb segment from chromosome 10 that includes the *SUN* gene for tomato fruit shape and its movement into the putative defensin gene *DEFL1* on chromosome 7 (Xiao et al., 2008). This movement allows the *DEFL1* promoter to drive *SUN*, leading to altered auxin levels (Xiao et al., 2008). This mutation has never been identified in a wild tomato, and it is most prevalent in Spanish varieties (Rodríguez et al., 2011), suggesting that the mutation appeared after the arrival of tomato cultivars in Spain.

Active transposons exist in crops, and it is probable that many traits selected during domestication were caused by transposable element movement. A prime example is yellow maize. The maize endosperm was originally white, due to the lack of phytoene synthase expression. A 382-bp *Ins2* insertion into the *yellow endosperm1* locus for phytoene synthase permits its expression in the endosperm, leading to the accumulation of carotenoids (Palaisa et al., 2003). Active transposons have also been found in some landraces of maize (de la Luz Gutiérrez-Nava et al., 1998; Fig. 3).

The full extent of transposon movement in modern crop varieties has not yet been determined, because the necessary genomic and bioinformatic tools for this analysis are just beginning to emerge. Crops with active transposons can have very high rates of transposition. The best characterized is the *Ping* element and its derivatives in rice (Nakazaki et al., 2003). Gimbozu, a historically important variety in Japan, shows approximately 50 to 60 new *Ping* insertions per plant per generation, occasionally resulting in phenotypic changes, such as the *slender glume* mutation (Nakazaki et al., 2003). Transposition in modern varieties bred from Gimbozu accounts for approximately one insertion per three plants per generation (Naito et al., 2006), so the movement of these transposons is likely widespread in farmers' fields. Given the long history of rice cultivation, it is noteworthy that there have been no reports of safety concerns from the insertion/excision of this element or any of the associated genomic changes.

Figure 3. Maize from a Bolivian landrace on Pariti Island, Lake Titicaca, shows evidence of transposable element activity. (Photograph by Eduardo Forno. This photograph may not be reproduced without the written permission of Eduardo Forno.)

Single-Base-Pair Changes and Indels Are Common in Plant Genomes

Single-base-pair differences between genomes are known as single-nucleotide polymorphisms (SNPs). A comparative analysis between 12 wheat varieties showed an average of one SNP per 540 bp (Somers et al., 2003). Soybean was found to have one SNP per 2,000 bp in coding regions and one per 191 bp in noncoding regions (Van et al., 2005). Rafalski (2002) compared polymorphisms between maize inbreds Mo17 and B73 and found one SNP per 130 bp within coding regions and one per 48 bp in 3′ untranslated genic regions. Tenaillon et al. (2001) estimated that two randomly chosen alleles of a maize gene encoding a protein of 300 to 400 amino acids would differ at 3.5 amino acids because of SNPs. Within a diverse population, there are likely 15 to 20 amino acid differences between proteins encoded by alleles of a single maize gene. In Arabidopsis, there is a seven in 1 billion chance that any given base pair will mutate in a generation (Ossowski et al., 2010). Given that there are 125,000,000 bp in the Arabidopsis genome, 1.75 new SNP mutations are expected per generation per diploid plant. Thus, 1,000 plants would have approximately 1,750 new base-pair mutations.

Indels are insertions or deletions of DNA in one DNA sequence relative to another. In maize, 43% of 215 loci examined had indels of 1 bp or more (Rafalski,

2002). Indels in the promoters of several rice genes alter their expression in the presence of certain transcription factor alleles, possibly resulting in hybrid vigor (Zhang et al., 2008). If gene promoters are affected, the timing and amounts of metabolites present in the plant may be altered, but novel compounds would not be produced.

Genomic Change through Mutation Contributes to New Plant Traits

Breeders sometimes induce mutagenesis to increase the level of genetic variation available for the selection of desired phenotypes. The Food and Agriculture Organization of the United Nations and the International Atomic Energy Agency maintain a database (http://mvgs.iaea.org/) listing 2,543 known plant varieties, including many common and widely grown crop plants, developed through radiation-induced mutagenesis (Ahloowalia et al., 2004). For example, gamma rays were used to generate a low-glutelin phenotype in rice. In this case, gamma rays caused a 130-kb deletion encompassing parts of two glutelin genes within the *glutelin1* locus (Morita et al., 2007). Gamma-ray-induced mutations in tree fruit have also been linked with insertions or deletions in known genes; for example, self-compatible apricot (*Prunus armeniaca*) mutants contain a 358-bp insertion in a pollen-expressed F-box gene (Vilanova et al., 2006), and two self-compatible cherry (*Prunus avium*) varieties have deletions in the same gene (Sonneveld et al., 2005). In many cases, the molecular basis for the altered phenotype of mutant varieties is unknown.

Genomic Changes and the Likelihood of Creating New Proteins

Genetic mechanisms that lead to changes in protein function and the evolution of gene families include (1) gene duplications, (2) divergence of gene sequences to produce proteins with modified structures and functions, (3) selection of new sequences with value to organism fitness, and, in many cases, (4) novel gene or exon combinations (Chothia and Gough, 2009). Gene duplications, combinations, and divergence occur across families of related genes and their encoded proteins (Perutz et al., 1960; Rossmann et al., 1974; Tang et al., 1978). Intronic recombination leads to more structurally complex proteins (Patthy, 1994). Ultimately, however, the creation of new biochemical function occurs gradually, because mutations are balanced by the requirement that any novel protein be properly folded, biochemically active, and useful to the organism. Proteins of various enzyme superfamilies with 20% to 50% sequence identity can share conserved active-site features and chemical reaction mechanisms yet exhibit wide ranges of substrate specificity and modified structural features (Jörnvall et al., 1995; Jez et al., 1997; Todd et al., 1999; Gerlt and Babbitt, 2001; Penning and Jez, 2001; Khersonsky et al., 2006; Redfern et al., 2008; Chothia and Gough, 2009). These studies demonstrate that evolution of a new enzymatic activity occurs within the constraints defined by the active site, the type of chemical reaction, and the substrates. Furthermore, there is a tradeoff between biochemical function and organism viability under a given selective pressure (Khersonsky et al., 2006), which limits possible changes.

Mutations in gene and protein sequences may be considered negative (leading to a loss of function), neutral (no change in function), or positive (leading to a new function). Analyses of sequence changes in proteins indicate that most mutations are deleterious and eliminated by natural selection (Wilson et al., 1977, 1987; Creighton, 1992). Similarly, directed evolution experiments using in vitro mutagenesis and selection strategies illustrate both the ability of a protein to accumulate multiple mutations without changes in function and the difficulty of modifying a protein to perform a new function (Arnold, 1993; Roodveldt et al., 2005; Gerlt and Babbitt, 2009; Tracewell and Arnold, 2009; Turner, 2009). For example, positive mutations that enhance weak catalytic efficiency by 10- to 1,000-fold are common; however, these experiments require many generations (rounds) of mutation and the targeted screening of millions of enzyme variants to arrive at a "new" or enhanced functionality.

CAN TRANSGENES ALTER GENOME STABILITY?

Transgene Instability

Transgenic DNA integration can sometimes be complex, involving the duplication of introduced sequences (Muskens et al., 2000). In general, single-copy transgenic insertions and their flanking regions are more stable than complex insertions with duplicated sequences (Papazova et al., 2006, 2008; Kohli and Christou, 2008), as the latter can undergo homologous recombination with resulting loss of function (Choffnes et al., 2001). In this regard, the behavior of multiple copies of a given transgenic insertion is similar to that of repetitive DNA in general or that of naturally occurring duplicated genes (e.g. the *I* and *a1* seed color loci described earlier). Because transgenes in plants are integrated into genomic DNA, they will change and recombine along with the rest of the genome. Species-specific differences in stability are extremely rare, with some genotypes of flax (*Linum usitatissimum*) being the main example (Cullis, 2005).

Some attention has focused on the 35S promoter from cauliflower mosaic virus, a promoter commonly used in commercialized GE crop plants. Ho et al. (2000) reported that this promoter contributes disproportionally to genomic instability; however, this conclusion is based on misinterpretation of the literature. The 35S promoter contains a recombination hotspot, associated with an imperfect 19-bp inverted repeat (Kohli et al., 1999), with the consequence that many transformants show rearrangements in the region (Kumpatla and Hall, 1999). Ho et al. (2000) overlooked

the fact that the reported rearrangements occurred in the plasmids used for transformation, not in the plants. Moreover, plant genomes have numerous inverted repeats of their own—wheat has at least 1 million such repeats and cotton has 40,000 (Flavell, 1985). Today, inverted repeats are frequently identified with microRNA genes, which play an important role in growth and development (Lelandais-Brière et al., 2010).

Because of the variability associated with transgenic DNA insertion, it is common for hundreds to thousands of transformation events to be screened to identify a single lead event intended for commercial release (McDougall, 2011). For example, over 1,300 initial transformation events were screened to identify the commercialized glyphosate-tolerant maize event NK603 (Heck et al., 2005). Events destined for commerce are thoroughly characterized for stable trait expression during breeding and are only advanced if the trait is stable over generations (Mumm and Walters, 2001). Molecular characterization of transgenic insertions is usually performed at early stages of event selection to remove those events with insertion arrangements (e.g. inverted repeats) that could affect trait expression (Mumm and Walters, 2001). Thus, single events lacking stability are identified early and not moved forward for further analysis.

To further evaluate the phenotypic stability of a lead event in a seed-propagated crop, field trials are conducted following multiple rounds of self-pollination or backcrosses into elite varieties (Padgette et al., 1995; Mumm and Walters, 2001; Heck et al., 2005). Such evaluations further help to uncover any stability concerns with a particular event before commercialization. In vegetatively propagated species and for species with long reproductive cycles, evaluation for stability in multiple environments or over multiple years serves a similar purpose. The breeding process further selects for events that are stably expressed irrespective of genetic background and of whether they are simple or complex loci (Mumm and Walters, 2001; Cellini et al., 2004). At the conclusion of the development process, a single event has been extensively evaluated for its phenotypic stability and, thus, for its genomic stability. Consequently, following the safety assessment, the lead single event destined for commercialization is expected to be as stable during breeding and propagation as any endogenous gene in a non-GE variety or hybrid. As an example, La Paz et al. (2010) evaluated the stability of the transgene for resistance to the European corn borer in the maize event MON 810, after 10 years of selective breeding across multiple genetic backgrounds, and could not find any evidence that the insertion or its flanking sequences were any less stable than those of endogenous genes.

Genome Instability

If transgenes are not more unstable than other genes in the genome, can they destabilize the genome as a whole? The only known mechanism whereby that could happen is by homologous recombination between two transgenes. The consequences depend on the location and orientation of the transgenes and are illustrated in Figure 2. In many cases, homologous recombination between two transgenes would result in large chromosomal rearrangements that would affect the plant's fertility and, thus, be eliminated from the population.

Transgenic Insertion Expression and Silencing in Genomes

Silencing of endogenous genes following transformation, and silencing of transgenes by other transgenes, have been observed (Matzke et al., 1989; Cigan et al., 2005). In some cases, gene silencing is an unintended outcome; other times, gene silencing is intentional. From a food and feed safety perspective, it is important to emphasize that, like genome instability, silencing is a natural phenomenon that is prevalent in all plants (Parrott et al., 2010).

The topic of gene silencing and its applications to crop improvement has been reviewed recently from a safety perspective for GE plants (Parrott et al., 2010). Both expression and silencing of the transgene are evaluated in the safety assessment of commercial events. GE stacks produced from safety-assessed single events are not expected to display increased expression variability and silencing compared with their parental lines, particularly if the transgenes in each event do not have sequences in common; nevertheless, such cases would be detected during the trait evaluation process that takes place prior to commercialization.

Persistence of Mutations

For a genetic change to become established in a population, it must occur in a cell that will eventually give rise to a gamete (Walbot, 1996). Genetic changes that are detrimental to the cell reduce fitness, reducing the likelihood that they will be transmitted to the next generation. If seeds are produced, then the seeds with the mutation are more likely to be less competitive with the other seeds derived from the population. Large genome changes such as inversions or translocations often decrease fertility, thereby reducing the chance that such changes will be passed on to subsequent generations or become established in a breeding population.

Occasionally, spontaneous mutations lead to desirable effects. For example, the horticulture industry has long used natural mutations (called bud sports) as a source of novel traits (Anonymous, 1920; Shamel and Pomeroy, 1936). For example, a novel grape (*Vitis vinifera*) genotype with different skin color is due to retrotransposon movement, and another is due to somatic recombination of two alleles following a double-strand break and its repair (Azuma et al., 2009). In many cases, the molecular basis for the altered phenotype of a mutant variety is unknown.

However, more often than not, heritable genomic changes cause a loss in uniformity of crop varieties

(Jensen, 1965). Therefore, to help maintain uniformity and yield potential, strict seed certification procedures were implemented early in the 20th century and remain in place today. Certified seeds are produced in isolation to minimize outcrossing and maintain seed purity, and off-type plants are diligently removed prior to seed set (Sleper and Poehlman, 2006; Acquaah, 2007).

The situation is somewhat different with farmer-saved seed, which usually is not subject to purity screening. Even if farmers engage in repeated cycles of seed saving, a mutation leading to a food or feed hazard would not increase in frequency unless it provided a selective advantage or was deliberately selected by the farmer. Although seed saving has been standard practice for centuries, there is no evidence of hazardous mutations that have accumulated unperceived by farmers. On the other hand, farmers at times intentionally select toxic crops that confer a benefit. For example, some farmers select cyanogenic cassava (*Manihot esculenta*) varieties over acyanogenic ones because the cyanogenic varieties suffer less pest damage and therefore yield more. Importantly, farmers are aware of the risks involved and take precautions during food preparation (Wilson and Dufour, 2002).

CONCLUSION: CAN TRANSGENES ALTER GENOME STABILITY TO COMPROMISE FOOD/FEED SAFETY?

The literature contains enough examples of spontaneous changes in plant genomes to permit inferences on the impact of these changes on the non-GE crop and on the food and feed safety of products derived from non-GE crops. In addition to the types of spontaneous genetic changes already discussed, many more undoubtedly are undetected in crops and wild populations. There is no evidence that links any genomic rearrangement to a novel food or feed health hazard. The Food and Agriculture Organization of the United Nations/World Health Organization (2001) recognize that conventional breeding practices in non-GE crops have increased gene and protein sequence diversity without any significant increase in the allergenic potential of food crops. Only a small fraction of proteins in food and feed are potential hazards as either toxins or allergens, and these fall into defined families related by both sequence and structure (Conner and Jacobs, 1999; Taylor and Hefle, 2001; Breiteneder and Radauer, 2004; Mills et al., 2004). Thus, neither changes in gene expression nor mutations in amino acid sequences are likely to alter the safety of a protein or lead to the production of novel metabolites. Thus far, there is no evidence that a random genomic change in a crop has ever resulted in a novel safety issue, even when new alleles or genes were created.

Because the molecular mechanisms leading to genomic changes are found in both non-GE and GE plants, and because there is no evidence or biological explanation to suggest that crops with different genome structures (e.g. type or amount of repetitive DNA) differ in genome stability, there is no reason to expect that the genome of a GE stack is less stable than that of a non-GE plant or of a GE plant containing a single event. Accordingly, the frequency of potential protein changes and the evolution of novel protein functions should not differ between a GE crop, whether a single event or stacked, and its non-GE version. Importantly, it should be noted that any rare recombination occurring between common regulatory (e.g. promoter) sequences in two transgenes will not yield a hybrid protein, since the common sequences are not part of the coding region (Fig. 2). Therefore, other than changes due to the transgene products, the risks of introducing new food hazards are no different from the risks associated with traditional breeding (Conner and Jacobs, 1999).

Even if any of the changes described here might pose a biosafety hazard, genomic changes in somatic cells have no lasting effect if they are not transmitted to progeny. Importantly, the plant containing the initial change must occur in a seed production field, not in a commercial grain production field, for the change to later be present at a significant level in food or feed products. Even then, the only way in which a rearrangement could be passed on to the progeny in any meaningful way is if such changes took place early in the seed production process and went undetected, which is unlikely given the methodologies employed to ensure uniformity and identity preservation during seed production.

The likelihood that any one mutation would create a biosafety issue is improbably small and would occur in a single plant in a field containing hundreds to millions of other plants. Thus, any negative consequences from that one mutation would be limited to seeds produced by that one plant, with dilution upon harvest minimizing the likelihood of any deleterious effects resulting from consumption. This large dilution factor helps explain why such changes, which may in principle lead to a negative effect, remain undetected and why breeding is generally considered a safe process.

Inasmuch as the stacking of different transgenic insertions sharing common genetic elements (e.g. promoters, coding sequences, or 3′ untranslated regions) leads to a marginal increase in the amount of repetitive DNA in a genome, there should be no significant instability above what is already present in the genome, since the majority of sequences in plant genomes are repetitive. Similarly, combining GE events with DNA sequences that are homologous to sequences in the host plant should not introduce measurable additional instability.

The weight of the evidence leads to the conclusion that enhanced genetic instability from a transgene or from common sequences in two or more transgenes is unlikely. Even then, the probability that any genetic instability will lead to an altered protein or metabolic product that creates a biosafety issue is exceedingly small; the production of a GE stack does not measurably increase this probability. There is no readily identifiable biological reason why genomic changes occurring in the breeding of a GE stack would be different in nature, scale, or frequency from those taking place in non-GE

crops or in GE crops with a single event. Silencing of transgenes caused by duplicate sequences is only of primary concern to commercial companies because the added value from the GE trait would be lost, but it poses no biologically realistic hazard otherwise. Therefore, evaluating transgenic insertion stability in a GE stack does not provide information that can contribute to its safety assessment. Instead, assessment should focus on whether interactions with adverse effects can occur in GE stacks (H.Y. Steiner, C. Halpin, J.M. Jez, J. Kough, W. Parrott, L. Underhill, N. Weber, and L.C. Hannah, unpublished data).

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Natalie Weber
Pioneer Hi-Bred International, Wilmington, Delaware 19880

Claire Halpin
University of Dundee at the James Hutton Institute, Division of Plant Sciences, Invergowrie, Dundee DD2 5DA, Scotland, United Kingdom

L. Curtis Hannah
University of Florida, Horticultural Science Department, Gainesville, Florida 32611

Joseph M. Jez
Washington University, Department of Biology, St. Louis, Missouri 63130

John Kough
United States Environmental Protection Agency, Washington, District of Columbia 20460

Wayne Parrott
University of Georgia, Center for Applied Genetic Technologies, Athens, Georgia 30602

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