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Understanding Plants Modified by Transgenesis Using High-Throughput Profiling Technologies and the Relevance for their Regulation in Canada

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ABSTRACT

Unintended effects can occur in transgenic plants through interactions between genetic elements at the site of insertion or through interactions between the introduced gene and endogenous plant processes. High through-put profiling technologies have been applied to identify unintended effects in transgenic plants and have provided insight into their nature and extent. These studies have revealed that the process of transgenesis is not universally disruptive to plants and does not necessarily produce unintended effects. In contrast, there is often significant variability in the profiles of plants that have been developed using conventional breeding techniques, suggesting that transgenesis is no more likely to produce unintended effects than these other breeding approaches. The nature of the transgene is likely to be the most important factor that determines the extent of unintended effects that occur in transgenic plants, although there is some evidence that even genes that alter transcriptional networks may not introduce unintended effects. These observations support Canada's unique regulatory approach, which is based on the presence of a novel trait as opposed to the process of transgenesis. This greater understanding of unintended effects in transgenic plants can also help further shape the regulatory system by guiding the allocation of resources to those transgenic plants that represent the greatest risk.

Keywords: plants with novel traits, pleiotropic effects, transgenic plants, safety, unintended effects
Abbreviations: CP4 EPSPS, CP4 5-enolpyruvylshikimate-3-phosphate synthase

CONTENTS

INTRODUCTION

In the development of transgenic plants, one area of concern has always been the introduction of unintended effects that could impact the safety of the plant (Kuiper *et al.* 2001; Kok and Kuiper 2003). Two types of unintended effects have generally been characterized (Miki *et al.* 2009). One type of unintended effect can arise through interactions between the inserted DNA and adjacent genomic DNA sequences. These effects are locus-specific and are referred to as position effects. The introduced transgene may introduce one or more phenotypic traits and the full range of these effects are known as the pleiotropic effects. While some of these may be the intended traits, other may be unintended and constitute the second type of unintended effect.

Current risk assessments use a targeted approach to identify unintended effects in transgenic plants. Important plant components and characteristics are analyzed to ascertain that levels of known toxins and allergens are not altered, the nutritional profile of the plant has not been affected and the agronomic performance of the plant has not changed. There has been some debate as to whether such targeted approaches will be able to identify all unintended effects (Cellini *et al.* 2004). High through-put profiling technologies are proposed to be a useful addition to current assessments because they would provide a non-targeted analysis of the transcriptomic, proteomic or metabolomic profile of the transgenic plant that will be more effective at identifying unintended effects (FAO/WHO 2000; Kuiper *et al.* 2001).

Despite the potential benefits of high through-put profiling technologies, they have not yet been adopted by regulatory authorities in the risk assessment of transgenic plants. However, a large number of studies applying these technologies to the study of transgenic plants have now been published. These studies have contributed substantially to our knowledge of the extent and nature of unintended effects in transgenic plants. In addition, this knowledge can now be examined in view of the history of cultivation of transgenic plants since transgenic plants have now been cultivated for thirteen years and they are currently being grown in 25 countries on 125 million hectares (James 2008).

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Invited Mini-Review

In this paper, studies of transgenic plants employing high through-put profiling technologies will be reviewed to summarize what we have learned about the potential for unintended effects to occur. In addition, the ways in which this knowledge supports current regulatory practices in Canada and may help shape them in the future will be discussed.

TRANSGENESIS DOES NOT NECESSARILY INTRODUCE UNINTENDED EFFECTS

One of the most important findings of high through-put profiling studies of transgenic plants is that transgenesis does not necessarily produce unintended effects. A number of high through-put profiling studies have accumulated at this point demonstrating that, besides the intended trait, transgenic plants can be highly similar to their non-transgenic comparators within the detection limits of the particular analytical method.

The absence of unintended effects has been demonstrated in plants with simple traits where either no novel protein is introduced or a single novel protein is introduced with no expected pleiotropic effects. For example, in a proteomic analysis using two-dimensional gel electrophoresis, hybrid tomato plants resistant to the tomato spotted wilt virus by an RNA-mediated mechanism did not show any differences when compared to unmodified hybrids (Corpillo *et al.* 2004). Similarly, a two-dimensional gel electrophoresis analysis of *Arabidopsis* plants expressing the *uidA* gene encoding *β*-glucuronidase demonstrated no unintended effects in the seed proteome (Ruebelt *et al.* 2006a), while a microarray study of developing wheat seeds with endosperm specific expression of an *Aspergillus fumigatus* phytase gene identified only small differences in gene expression that could be attributed to asynchronous development of wheat seeds (Gregersen *et al.* 2005). Transgenic soybean expressing CP4 5-enolpyruvylshikimate-3-phosphate synthase (CP4 EPSPS),which confers tolerance to the herbicide glyphosate, also showed no significant differences in the proteome compared to a non-transgenic control when assessed using capillary electrophoresis-time of flight mass spectrometry (Simó *et al.* 2010).

Plants with more complex metabolic engineering traits have also been shown to be highly similar to their non-transgenic counterparts using high through-put profiling technologies. No unintended effects were revealed in the seed proteome of *Arabidopsis* plants engineered for increased levels of tocopherol through introduction of either a gene encoding *p*-hydroxyphenylpyruvate dioxygenase (*hppd*) or a gene encoding *γ*-tocopherolmethyl transferase (*gtmt*) (Ruebelt *et al.* 2006a). Similarly, *Arabidopsis* plants expressing three enzymes that constitute a dhurrin biosynthesis pathway demonstrated no unintended effects outside of the production of dhurrin using a combined microarray and metabolomic analysis (Kristensen *et al.* 2005).

Many of these studies were conducted using controlled growth environments in order to limit the effects of environmental variability. However, it is possible that unintended effects may only be produced under less optimal growth conditions. The extension of these studies to plants grown under less optimal growing conditions is therefore necessary to provide further support that transgenesis does not necessarily result in unintended effects. This was demonstrated by Baker *et al.* (2006) in a study on field-grown transgenic wheat expressing additional copies of genes encoding high-molecular-weight subunits of glutenin. These plants showed no reproducible differences in the grain metabolome when compared to a control parental line and a null transformant as determined following multivariate comparison of the proton nuclear magnetic resonance spectra of polar metabolites. Another study has examined whether unintended effects may be revealed under stress conditions. *Arabidopsis* plants expressing two common selectable markers, *nptII* and *uidA*, demonstrated no significant differences in the transcriptome under optimal conditions and these plants responded similarly to abiotic stresses such as salt, drought, cold and heat (El Ouakfaoui and Miki 2005).

These studies support the general conclusion that the process of transgenesis is not universally disruptive to the plant. Specifically, the insertion of exogenous DNA into a plant genome does not necessarily affect global gene expression patterns or protein and metabolite profiles. These studies included transgenic plants produced through both *Agrobacterium*-mediated and particle bombardment transformation methods indicating that this conclusion can be made for both transformation methods. In striking contrast to the above studies, the application of high through-put profiling studies to examine the stress response in plants has revealed large alterations in transcriptomes (El Ouakfaoui and Miki 2005; Kawaura *et al.* 2006; Lian *et al.* 2006), proteomes (Majoul *et al.* 2003; Cui *et al.* 2005; Hajheidari *et al.* 2005) and metabolomes (Kaplan *et al.* 2004; Charlton *et al.* 2008). Were transgenesis to impose a stress on the plant, similarly large alterations would have been observed in the above studies. This is perhaps not surprising in the context of the dynamic nature of plant genomes. Plant genomes, even within a single species, can vary extensively in content and organization and they are constantly undergoing rearrangements due to the activity of transposons and retrotransposons (Cellini *et al.* 2004; Bradford *et al.* 2005).

These results have also provided important support for the Canadian regulatory system. In Canada, the regulation of transgenic plants is trait-based not process-based, which is in contrast to the regulatory approach of other countries. Basically, a risk assessment is triggered not by the fact that a plant is transgenic but by the fact that it possesses a novel trait not present in the Canadian environment or in approved foods and feeds. These high through-put profiling studies support this approach because they demonstrate that unintended effects do not necessarily arise from the process of transgenesis itself.

These studies have also been valuable for demonstrating that commonly used selectable markers such as *nptII* and *gus* do not produce unintended effects. Selectable markers are often present in transgenic plants intended for unconfined release and this knowledge helps to support the safety of these genes and could in the future help to reduce the need for a full safety assessment for these genes. This knowledge is also essential for research in functional genomics, which often involves the use of transgenic plants, to ensure that results are correctly interpreted (Miki *et al.* 2009). It is also possible for selectable markers to be associated with unintended effects. For instance, microarray analysis of *Arabidopsis* plants expressing the *bar* gene, which confers resistance to the herbicide glufosinate, revealed that four genes were differentially expressed compared to non-transgenic plants. In response to glufosinate application, 29 genes were uniquely differentially expressed in the transgenic plants expressing *bar* (Abdeen and Miki 2009). In such cases, these differences could be taken into consideration in the analysis of transgenic plants expressing the *bar* selectable marker, if a potential risk could be identified from the analysis.

More recently, a microarray analysis has also been used to assess the potential for unintended effects to occur from the expression of the *Cre* gene encoding Cre recombinase (Abdeen *et al.* 2010). Recombinases such as Cre can be used for the site-specific integration of transgenes and they can also be used to excise unnecessary genetic elements from the inserted DNA such as selectable markers (Gilbertson 2003). They have the potential to provide even greater precision to the introduction of DNA into plant genomes, but there is also some concern about their potential to cause unintended effects by mediating recombination within the plant genome (Gilbertson 2003). Microarray analysis of three independent *Arabidopsis* lines from which transgenes were removed through Cre-mediated excision revealed only a small number of genes that were differentially expressed (Abdeen *et al.* 2010). Two of these genes were common to

two of the three plant lines and both were encoded by the chloroplast genome. These results demonstrated that unintended effects generated by Cre recombinase were minimal, and potentially limited to the chloroplast genome. This study helps to extend the conclusion that transgenesis does not necessarily result in unintended effects to include recombinase-mediated genetic manipulations.

UNDERSTANDING NATURAL VARIABILITY

High through-put profiling studies have illustrated that there is a high degree of natural variability in plants. Even small differences in genotype and environment can impact phenotype, and these differences will be reflected by variations in the levels of transcripts, proteins and metabolites. For example, amongst 12 ecotypes of *Arabidopsis*, 597 spots out of a total of 931 spots detected were absent in at least one ecotype and amongst those spots equally expressed in all ecotypes, 95% varied in spot quantity with differences varying from 2-fold to 53-fold (Ruebelt *et al.* 2006b). Variation can also be observed within an ecotype as seeds of the same Columbia-0 ecotype of *Arabidopsis* obtained from two different sources showed small differences in the proteomic profile (Ruebelt *et al.* 2006a). This natural variability can be a limitation to high through-put profiling studies by occluding those differences that can be attributed to the presence of the transgene, necessitating carefully designed experiments in order to limit variability. However, the natural variability can also be a useful benchmark for determining which differences are significant.

In some cases, environmental variability has been used as a measure of natural variability in high through-put profiling studies of transgenic plants. This was done in the field study of transgenic wheat expressing the HMW subunits of glutenin by Baker *et al.* (2006). As already mentioned, these plants were determined to be highly similar to their non-transgenic counterparts with the exception of the intended trait and this conclusion was based on the fact that any observed differences were within the range of differences that were observed in different site-year combinations.

Barros *et al.* (2010) had a similar observation when comparing transgenic maize expressing either Cry1Ab from *Bacillus thuringiensis* or CP4 EPSPS to a near-isogenic cultivar. Several differences were found between the transgenic and non-transgenic plants including lower levels of expression of the maize allergen Zea m14 in both transgenic lines, increased levels of glucose and fructose in the line expressing Cr1Ab, and lower levels of γ-tocopherol and inositol in the line expressing CP4 EPSPS (Barros *et al.* 2010). However, in contrast, differences in gene expression, proteomic and metabolic profiles across growing seasons were much larger than those observed between the transgenic and non-transgenic plants (Barros *et al.* 2010).

Another approach for defining the range of variability is to include a range of different cultivars in addition to the parental line as non-transgenic controls. Components identified as different must therefore lie outside of the range of values for that component from the different cultivars assessed. This is a similar approach to the targeted safety assessment of transgenic plants adopted by many national regulatory authorities. Databases (e.g. the ILSI crop composition database; www.cropcomposition.org) provide species-specific information about the important components and a range of variability assessed in a number of genotypes. This approach was taken in a metabolomic study by Catchpole *et al.* (2005) on transgenic potatoes expressing one or two enzymes involved in the synthesis of inulin-type fructans. Using this approach, the only metabolites with levels outside the range established by the comparative cultivars were those associated with the introduced trait.

This approach may be especially useful when a near-isogenic line is not available for comparison with the transgenic plant. In a study by Cheng *et al.* (2008), two separate transgenic glyphosate-resistant soybean expressing CP4 EPSPS, both descended from the same original transformation event, were compared to three different conventional cultivars. When the transgenic lines were compared to their closest genetic relative from amongst the three conventional cultivars, 44 and 109 genes were identified as being significantly differentially expressed. However, when compared to all three conventional cultivars, only 10 and 49 genes were identified as significantly differentially expressed.

Using natural variability as a benchmark in this way, it is possible that some differences will not be identified because they fall within the range of natural variability but are in fact true unintended effects. However, such unintended effects are unlikely to be significant from a safety perspective precisely because they are within the range of natural variability for that plant species. This approach is therefore effective at specifically identifying those unintended effects that may be of significance from a safety perspective.

As part of meeting the need to define the range of variability for high through-put profiling data, the call to create databases has been made (Cheng *et al.* 2008). These would be equivalent to the compositional databases developed for the targeted assessments. It would help simplify the analysis done on each new trait by removing the need for multiple comparators to be included in the analysis. However, there will be a number of challenges to overcome in the creation of such databases to ensure that the data submitted is consistent across the various studies. In particular, the effects of growth conditions, developmental age, time of day as well as the statistical methods used can all significantly impact the results of such studies and a rigorous approach will be needed to standardize results. Should regulatory bodies decide to require the use of high through-put profiling studies for the assessment of transgenic plants, this will be a significant hurdle to overcome. It would be essential to define the acceptable range of variability for each transcript, protein and metabolite in order to assess the significance of identified differences. It can be anticipated that this would require a great amount of time and resources in order to gather this breadth of information for all the currently grown major crops. Alternatively, it may be possible to gather such information for a smaller subset of components that could act as indicators of unintended effects. However, this approach would suffer similar restraints as targeted approaches, since it would rely on our ability to predict what unintended effects may be significant from a safety perspective.

CONVENTIONAL BREEDING TECHNIQUES VERSUS TRANSGENESIS

High through-put profiling studies have also revealed some interesting differences in the potential for unintended effects to occur in transgenic plants versus plants produced through conventional breeding techniques. For instance, an extensive two-dimensional gel electrophoresis analysis of the proteome profile of different potato genotypes, including 21 tetraploid cultivars and 8 landraces of *Solanum tuberosum* and 3 diploid genotypes of *Solanum phureja*, found that 1077 spots out of 1111 showed significantly different expression amongst the genotypes (Lehesranta *et al.* 2005).

This was similarly demonstrated in a study by Baudo *et al.* (2006), which compared differences between two wheat sister lines and a transgenic plant and its parent. The two wheat sister lines were selected from the F2 progeny derived from a cross between two different cultivars and one of the sister lines was subsequently transformed with a high molecular weight subunit of glutenin. Microarray analysis was performed on endosperm tissue at 14 and 28 days post anthesis and on leaf tissue at 8 days post germination. The analysis revealed far greater differences in expression between the two sister lines (0.27-0.99% of genes) than between the transgenic plant and its parent (0.02-0.06% of genes) (Baudo *et al.* 2006).

Evidence for the influence of breeding on global gene expression is particularly evident in the study by Kogel *et al.*

(2010). Comparison of two barley cultivars, Baronesse and Golden Promise, revealed 1,660 genes with differential expression. Prominent amongst these genes were many associated with defense responses, which is likely reflective of selection during breeding for disease resistance (Kogel *et al.* 2010). In contrast, comparison of transgenic lines derived from these two cultivars revealed fewer changes. Plants expressing an endochitinase from *Trichoderma harzianum* had no difference in either the gene expression or metabolic profile when the plants were compared to Golden Promise (Kogel *et al.* 2010). Although plants expressing a (1,3-1,4)-β-glucanase did have 22 differentially expressed genes and 4 altered metabolites compared to Baronesse, most of the differences were thought to be the result of differences in the genetic background of the transgenic plant and the comparator since the trait was introgressed from the Golden Promise background into the Baronesse background (Kogel *et al.* 2010).

A microarray study by Batista *et al.* (2008) demonstrated that mutagenesis can result in greater perturbations to gene expression profiles than transgenesis in rice. In mutational breeding, in addition to the desired mutation, it is likely that there will be extensive background mutations. In a study in which the wheat genome was mutagenized by ethyl methane sulphonate for TILLING, it was estimated that each individual could carry 260,000 mutations if tetraploid and 415,000 mutations if hexaploid. While many of these mutations would have little impact due to the presence of repetitive elements and the low gene density of the wheat genome, it was still estimated that there could be thousands of missense mutations and hundreds of mutations resulting in truncations (Uauy *et al.* 2009). Background mutations have the potential to produce unintended effects in plants similar to transgenic techniques and the study by Batista *et al.* (2008) demonstrates that such effects are in fact quite likely. Many of the background mutations can be eliminated through backcrossing but it would be difficult to confirm their complete absence.

These studies demonstrate that conventional breeding techniques generally result in greater differences in the transcriptomic, proteomic and metabolomic profiles of the plants than do transgenic techniques. This supports the conclusion that unintended effects are as likely to occur in new plant varieties produced using conventional breeding techniques as they are to occur in transgenic plants (Kuiper *et al.* 2003; Cellini *et al.* 2004) and further demonstrates that they are perhaps even more likely to occur with conventional breeding techniques. These findings suggest that new traits can be introduced into plants with greater precision through transgenesis than through conventional breeding techniques. The development of new technologies, such as the use of recombinases (Ow 2002) and zinc finger nucleases (Shukla *et al.* 2009) for site-specific transgene integration and the use of artificial minichromosomes (Ananiev *et al.* 2009), has the potential to further enhance the precision of transgenesis.

It is important to note that plants produced with conventional breeding techniques have not typically been required to undergo a rigorous risk assessment. As already discussed, the Canadian regulatory system is trait-based not process-based. The Canadian system is therefore unique in that the same regulatory constraints are placed on plants derived through conventional breeding as those produced through transgenesis. Essentially, any plant with a novel trait is required to undergo a risk assessment regardless of the mechanism through which the novel trait is introduced. Novel traits introduced through conventional breeding approaches are subject to the same regulatory constraints as those produced through biotechnology. High through-put profiling studies have supported this approach by demonstrating that the risk imposed by transgenesis is no greater than the risk of plants produced through other techniques.

UNDERSTANDING UNINTENDED EFFECTS

Some of the high through-put profiling studies of transgenic crops have also identified differences between transgenic crops and their comparators that may represent unintended effects. These studies have validated the ability of these technologies to detect such differences. These studies have also generated some knowledge of the ways in which the introduction of novel genes can alter plant processes. Such knowledge can contribute to refining the development of transgenic plants in the future in order to minimize unintended effects.

High through-put profiling studies have demonstrated the importance of the rational engineering of plants. As already discussed, the introduction of all three enzymes of the dhurrin biosynthesis pathway from *Sorghum bicolour* did not result in any unintended changes to the transcriptome or metabolome (Kristensen *et al.* 2005). However, the introduction of the first two enzymes of the pathway without the third enzyme produced a number of unintended effects, including the accumulation of glucosides from the detoxification of intermediates in the dhurrin biosynthesis pathway as well as the unpredictable loss of the UV protectants sinapoyl glucose, sinapoyl malate and kaempferol glucosides, all of which were accompanied by several alterations in the gene expression profile (Kristensen *et al.* 2005). This study demonstrates that a careful understanding of metabolic pathways may help guide metabolic engineering in order to minimize unintended effects. In this case, the introduction of a complete pathway for the production of a single endpoint metabolite prevented the accumulation of unwanted intermediate metabolites.

In another study, the introduction of a single novel enzyme had a number of effects on plant metabolism. Simoh *et al.* (2010) introduced a gene encoding isochorismate synthase from *Escherichia coli* into *Brassica rapa* ssp. *oleifera* in order to increase the production of salicylic acid. Analysis of the plants by 1-dimensional proton nuclear magnetic resonance revealed increases in the glucosinolate-neoglucobrassicin, the phenylpropanoids sinapoyl malate, feruloyl malate, and caffeoyl malate, the organic acids succinic acid and fumaric acid, and α- and β-glucose while levels of amino acids, including alanine, threonine, valine, and leucine were decreased compared to controls. Isochorismate synthase uses chorismate as a substrate and these results suggest that its introduction affected other metabolic pathways that also compete for this substrate (Simoh *et al.* 2010).

Other studies have identified unexpected physiological responses to the introduction of a novel protein that resulted in unintended effects. A proteomic study of rice endosperm cells expressing human granulocyte-macrophage colony stimulation factor revealed 103 of 1883 proteins with significant differences compared to the non-transgenic equivalent (Luo *et al.* 2009). A number of the proteins showing up-regulation were identified as being either molecular chaperones or related to the 26S proteasome. These results suggested that the expression of the human granulocyte-macrophage colony stimulation factor may be stimulating the unfolded protein response, which triggers the expression of molecular chaperones and activation of endoplasmic reticulum-associated degradation activity mediated by the ubiquitin-proteasome pathway. Further analysis supported this hypothesis (Luo *et al.* 2009). This suggests that expression of a recombinant protein has the potential to trigger the unfolded protein response in plants.

In another study on a transgenic wheat line overexpressing a low molecular weight glutenin subunit, a downregulation of the majority of storage proteins from the prolamin family was observed based on microarray analysis of developing seeds and proteomic analysis of flour from mature seeds (Scossa *et al.* 2008). It was suggested that this effect was most likely related to a compensatory mechanism sometimes observed in plants where silencing or degradation pathways are activated in order to prevent the overaccumu-

lation of storage reserves. The implications of such effects may be varied since it may reduce the levels of certain endogenous allergens, which would in fact be a beneficial unintended effect (Scossa *et al.* 2008); however, changes to the overall nutritional quality of the flour would need to be considered and could potentially be undesirable.

The above studies demonstrate unintended effects that result from the expression of a novel protein. The identification of such effects provides an opportunity to further study them. Knowledge gained from such study can then be applied to limit their occurrence in the development of other transgenic plants. Knowledge of these types of physiological responses can also help in the development of screening methods in order to detect plants in which such responses are occurring and omit them from further development.

The application of high through-put profiling technologies to the study of transgenic plants has also identified a challenge in the analysis of unintended effects. This challenge is determining which significant differences are in fact significant from a biological perspective as it applies to the safety of transgenic plants. The identification of differences does not in and of itself identify a hazard. Instead, it represents a starting point for further risk assessment. In the study by Baker *et al.* (2006), one of three transgenic wheat lines showed differences in levels of the disaccharides maltose and sucrose, two common and abundant metabolites, at four of the six site-year data sets, although these differences were within the range of environmental variability from different site-year combinations (Baker *et al.* 2006). While the altered levels of maltose and sucrose in the transgenic wheat plants may affect the nutritional profile of the plant, they are unlikely to impact the safety of the plant. In contrast, a metabolomic study done on transgenic rice expressing *cryIAc* and *sck* genes, both of which confer resistance to Lepidoptera insects with the *sck* gene also conferring resistance to Coleopteran insects, identified a number of differences when compared to a non-transgenic counterpart, including increases in the levels of sucrose, mannitol and glutamic acid (Zhou *et al.* 2009). Similarly, two studies done on a maize cultivar into which the MON810 *cry1Ab* insertion event had been introgressed identified a number of differences. A proteomic analysis revealed the unique presence of a glucose and ribitol dehydrogenase and the absence of endochitinase A as well as an increase in the levels of triosephosphate isomerase 1 and globulin-1 S and a decrease in the levels of cytosolic 3-phosphoglycerate kinase and aldose reductase (Albo *et al.* 2007). Variations in the levels of osmolytes and branched amino acids were detected in a metabolomic analysis (Manetti *et al.* 2006). Interestingly, other studies have come to conflicting conclusions for MON810, identifying no significant differences in the transcriptome (Coll *et al.* 2009) and the proteome (Coll *et al.* 2011), while others identified other unintended effects in the transcriptome (Barros *et al.* 2010), proteome (Zolla *et al.* 2008) and metabolome (Levandi *et al.* 2008; Piccioni *et al.* 2009; Barros *et al.* 2010).This is likely reflective of differences in experimental design, analytical methods and genetic backgrounds in the various studies. In cases like these, it is less clear what impact the altered metabolites and proteins would have in the rice plants expressing *cryIAc* and *sck* or the maize plant expressing *cry1Ab*. Further analysis would be necessary to determine the risk of such differences.

It is also important to consider that many observed unintended effects may be associated with phenotypic abnormalities that would prevent such plants from ever being considered for commercialization. An interesting metabolomics study done in potato looked at a range of different transgenic events that produced alterations in either metabolism or developmental processes with a range of phenotypes (Defernez *et al.* 2004). In this study, the tubers showing the greater number of differences in the metabolomic profile originated from plants with the more severe phenotype. The plants showing the greatest number of changes were those expressing an antisense S-adenosylmethionine decarboxylase, which exhibited stunted growth as well as reductions in dry matter content and the number of tubers. Those showing an intermediate number of changes included plants showing downregulation of the endogenous granule-bound starch synthase gene as a result of co-suppression through the introduction of a granule-bound starch synthase promoter, as well as plants expressing an antisense copy of the *MAL1* gene, which encodes a glycoprotein-processing type II enzyme. These plants showed a waxy phenotype related to high amylopectin content and stunted growth along with altered leaf morphology, respectively. Overall, even those changes identified in the plant lines with the most severe phenotype were not large and differences observed between the two non-transgenic cultivars were in fact greater. Plants with such severe phenotypes would not even be considered for further development since they show characteristics that are not desirable. Therefore, this study suggests that in many cases such screening would in fact be effective at eliminating those plants showing major changes to the transcriptome, proteome or metabolome. A similar relationship between unintended effects detected by high through-put profiling studies and phenotypic abnormalities was also seen in the study by Kristensen *et al.* (2005), where introduction of part of the dhurrin biosynthesis pathway introduced alterations in the transcriptome and metabolome but also resulted in a stunted phenotype.

While high through-put profiling technologies have undoubtedly proven effective at detecting unintended effects in transgenic plants, the one element that is missing in proving their usefulness is to demonstrate that identified differences are both of relevance to the safety of transgenic plants and outside the range of detection of the currently employed targeted methods. Given the already lengthy assessments that are applied to transgenic plants, it would be inefficient to introduce new constraints to apply high through-put profiling technologies that will detect a number of differences, each of which must be fully assessed for safety, but few of which have any actual potential to pose a risk. Given the dynamic nature of plant genomes and the high degree of variability that has been found in transcript, protein and metabolite profiles, it is likely that many of the detected differences will be irrelevant to the safety of the transgenic plants.

With the example of the MON810 trait, it is now possible to consider the observed unintended effects of the insertion event (Manetti *et al.* 2006; Albo *et al.* 2007; Levandi *et al.* 2008; Zolla *et al.* 2008; Piccioni *et al.* 2009; Barros *et al.* 2010) in view of a history of safe use. MON810 was approved in Canada for unconfined release as well as use as food and feed in 1997. As a result, there is now a history of safe use for this novel trait in maize of over a decade. This would suggest that the unintended effects that were identified are unlikely to impact the safety of the transgenic maize.

UNINTENDED PLEIOTROPIC EFFECTS

Those transgenic plants that have reached the market have predominantly been those with either herbicide tolerance or insect resistance. Both of these traits are considered to be simple, since they are associated with the introduction of a single gene that is expected to produce a single novel protein that will impart a novel trait without interacting with any endogenous plant processes. However, it has been predicted that transgenic plants with more complex traits, particularly abiotic stress tolerance and enhanced yield, will soon be introduced (Century *et al.* 2008). Such traits are usually achieved specifically by altering, often extensively, endogenous plant processes. It can therefore be expected that large changes in the transcript, protein and metabolite profiles may be identified. For example, it is anticipated that many of these traits will be engineered through the modification of transcription factors (Century *et al.* 2008) and therefore the desired effect is the modulation of gene expression. With such traits, there is some concern that

unintended pleiotropic effects may be more likely to occur. Many of these key regulatory genes, such as transcription factors, may have multiple functions, depending on the timing and location of their expression. Altering their pattern of expression may therefore reveal unexpected functions that result in unintended effects.

Despite these concerns, a microarray analysis of drought-tolerant *Arabidopsis* plants overexpressing the transcription factor *ABF3* demonstrated that such an approach may not necessarily produce unintended effects. In the absence of stress, overexpression of *ABF3* did not affect the gene expression pattern. During drought stress, differences were observed in the pattern of gene expression in these plants but these differences more reflected the modulation of the drought response due to the overexpression of *ABF3* and did not appear to relate to unintended effects (Abdeen *et al.* 2010).

It is interesting to consider that in the case of abiotic stress tolerance, such traits may in fact maintain the standard of composition more effectively than their non-transgenic counterparts when suboptimal growing conditions are imposed. Abiotic stresses will often alter a plant's composition (Dornbos and Mullen 1992; Dakhma *et al.* 1995; Carvalho *et al.* 2005; Rotundo and Westgate 2009), in some cases resulting in the production of toxins and anti-nutrients (Champolivier and Merrien 1996; Jensen *et al.* 1996; Bejarano *et al.* 2000) for which transgenic plants are so carefully screened. The introduction of abiotic stress tolerance may contribute to the prevention of such undesirable alterations. In such cases, the transgenic counterpart may actually be deemed as more safe than the non-transgenic counterpart as opposed to the standard that is typically sought, which is that transgenic plants be only as safe as their non-transgenic counterpart.

While the work of Abdeen *et al.* (2010) demonstrates that some of the more complex traits may not necessarily produce unintended effects, such results will likely depend on the specific gene introduced, continuing to warrant a case-by-case approach to the regulation of transgenic plants. In fact, a number of studies have demonstrated that overexpression of transcription factors can impact the transcriptome (Li *et al.* 2004; Yi *et al.* 2005; Nelson *et al.* 2007; Batista *et al.* 2008). One of the challenges for these types of modifications is determining which differences relate to the intended trait and which are unintended.

It has been suggested that transgenic plants could be classed into groups with different levels of anticipated risk based on the nature of the transgene (Bradford *et al.* 2005). This would enable resources to be focused on those transgenic plants representing the greatest level of risk. The results of high through-put profiling studies support this suggestion. Those transgenes that introduce a simple monogenic trait such as *nptII* and *gus* (El Ouakafaoui and Miki 2005; Ruebelt *et al.* 2006a) did not produce unintended effects. Metabolic engineering in some cases did not result in unintended effects (Kristensen *et al.* 2005; Ruebelt *et al.* 2006a) while in other cases it did (Defernez *et al.* 2005). More complex traits that involve manipulating endogenous plant processes will not necessarily produce unintended effects (Abdeen *et al.* 2010) but may still be more likely to do so. These observations may provide justification to help streamline the risk assessment of transgenic plants in the future.

CONCLUSIONS

While unintended effects can occur in transgenic plants, the results of high through-put profiling studies of transgenic plants suggest that in many cases they do not. Certainly, the process of transgenesis is not stressful for plants as this would be reflected by numerous changes to the transcriptome, proteome and metabolome. In fact, in contrast to conventional breeding techniques, transgenesis appears to be less likely to produce unintended effects, although this may in part depend on the nature of the transgene. The absence of unintended effects that has been demonstrated in a number of transgenic plants to date would also suggest that position effects may be a rare occurrence that has only a minimal impact on the plant. Unintended pleiotropic effects of the transgene may require further consideration as they will depend in large part on the nature of the transgene. These conclusions support Canada's unique regulatory system that is based on the presence of a novel trait as opposed to the process of transgenesis. Through understanding unintended effects in transgenic plants, it may also be possible in the future to tailor the regulatory system in order to put more resources towards those plants that present the greater risk.

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