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ORIGINAL ARTICLE

Opportunities and surprises in crops modified by transgenic technology: metabolic engineering of benzylisoquinoline alkaloid, gossypol and lysine biosynthetic pathways

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Abstract The development of new or improved traits in plants, whether that is through traditional genetic modification and selection or through transgenic technologies, is associated with the potential risk of unintended changes with harmful or unacceptable consequences. The greater definition and precision of transgenic modification and the regulatory oversight of such technology may, however, confer advantages in safety and efficacy. This bears considerable relevance to the use of transgenic-based metabolic engineering in agricultural trait development. Metabolic engineering seeks to modify the amounts or chemical structures within selected biosynthetic routes without introducing inadvertent effects on other metabolic pathways. Examples discussed here include attempts to; (i) modify benzylisoquinoline alkaloid biosynthesis in poppy, (ii) improve the nutritional value of maize by increasing levels of free lysine, and (iii) increase the nutritional value of cottonseed by eliminating gossypol production. Clearly, evaluation of the efficacy (and unintended consequences) of such approaches is vital. A role for metabolomics in the compositional and metabolite analyses of new plant varieties derived from transgenic-based metabolic engineering is discussed. Major themes discussed in this review include; (i) the heightened level of scrutiny associated with genetically modified (GM) crop evaluations has markedly contributed to the safety in the adoption of transgenic technology, and (ii) the nature of any introduced trait may prove more relevant to safety assessments than the means by which the trait is introduced.

Keywords Benzylisoquinoline alkaloids · Gossypol synthesis · GM crops · Lysine biosynthesis · Metabolic profiling · RNAi

1 Introduction

Genetically modified (GM) crops withstand rigorous compositional, nutritional and safety evaluations prior to commercialization. In contrast, new plant varieties developed through conventional breeding are typically, but not always, exempt from such detailed evaluation. This difference in approach exists despite the fact that conventional (non-GM) crops display remarkable genetic plasticity and selective breeding approaches to elicit trait improvements are particularly susceptible to unintended phenotypic and metabolic effects. Crops genetically modified using traditional techniques including irradiation, chemical mutagenesis and selective breeding are considered to have a history of safe use. To date, literature reports spanning over a decade consistently highlight the compositional similarities of GM crops with their conventional counterparts. These studies include assessments of soy (Padgette et al. 1996; Taylor et al. 1999), corn (Ridley et al. 2002; Sidhu et al. 2000; Herman et al. 2004), cotton (Bertrand et al. 2005; Nida et al. 1996), rice (Oberdoerfer et al. 2005), wheat (Obert et al. 2004), and alfalfa (McCann et al. 2006)). Recent literature surveys (Preston 2004; Flachowsky et al. 2005; Flachowsky et al. 2007) of peer-reviewed feeding studies have also concluded that the first cohort of released GM crops are as safe, if not safer, than non-GM crops.

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The promotion of safety conferred by regulatory oversight of GM crops has led others to suggest that non-GM crops, particularly those bearing novel traits, should be considered subject to similar scrutiny. As stated by Cellini et al. (2004), "Unintended effects occur in both GM and non-GM crops; however, GM crops are better characterized. It may be suggested that the two should be treated the same in safety assessments, bearing in mind that safety assessments are not required for non-GM crops." To illustrate the types of concerns associated with a differential approach to evaluations of GM and non-GM crops, consider the following two examples.

(a) Conventional cultivars of tomato have been bred to be resistant to nematodes through the introduction of genes from a related but poisonous South American species, *Lycopersicon peruvianum.* Through conventional means, a fragment of the *L. peruvianum* chromosome carrying the resistance gene, *Mi*, has been introduced. However, probably hundreds of other unknown *peruvianum* genes are now present in these new tomato cultivars through incorporation of the *Mi*-bearing chromosome fragments. *L. peruvianum* is not in the human food chain but most countries place no restrictions on the release of the new varieties. By contrast, consider that the specific *Mi* gene has been identified, isolated and cloned (Milligan et al. 1998). Indeed, the *Mi* gene has now been selectively introduced into a conventional tomato and does confer nematode resistance (Rossi et al. 1998; Vos et al. 1998; Goggin et al. 2006). The nutritional and agronomic effects of the Mi trait can now be specifically assessed, and the protein encoded by the *Mi* gene can be tested directly and exhaustively for toxicity and allergenicity effects. Most people given this information arrive at the view that the greater inherent risk resides, not with the GM tomato, but with the conventionally bred tomato, carrying hundreds of unknown genes from a poisonous and inedible plant and not subject to regulatory scrutiny.

(b) Barley Yellow Dwarf Virus (BYDV) is, economically, the world's most significant viral disease of small grain cereals including wheat, barley and oats. The CSIRO laboratories have developed resistant wheat cultivars by introducing a fragment of chromosome from a perennial grass, *Thinopyrum intermedium* (Banks et al. 1995). This type of chromosomal manipulation has been utilized frequently in conventional breeding, especially in the cereals. Levels of resistance are very good; however the introduced resistance comes with the "baggage" of an estimated 400 other genes whose identity and function are totally unknown. *Th. intermedium* is not a human food but, in most countries, there are no restrictions placed on the release of the new wheats. This situation can be contrasted with the GM solution to the same problem. An artificial gene producing hairpin RNA complementary to a fragment of one gene of the virus has been transferred to both wheat and barley where it results in excellent levels of BYDV resistance through the RNAi mechanism (Wang et al. 2000; Abbott et al. 2002). In any prospective commercial transgenic it is required to analyze where the sequence has been inserted and ensure that it does not disrupt other genes. No such evaluations are required for traditionally developed varieties of BYDV resistant wheats; however the addition of hundreds of genes with unknown functions may represent greater inherent risk to environment or health.

In many cases to date, GM crops have incorporated a discrete protein entity encoding a defined bioactive trait, typically insecticidal activity or herbicide resistance. A well known example is the *Bacillus* sp. cry1A(b) protein which, when incorporated into plants, confers insect-resistance (Koziel et al. 1993). Options exist however to modify entire biosynthetic pathways through mutation or modified expression of component enzymes. In some respects transgenic modification of a specific pathway may be considered more complex and more prone to unintended consequences than incorporation of, for example, a single insecticidal protein. Yet metabolic engineering through transgenic technologies also offers new opportunities to alter the specific content of important plant-based secondary metabolites, including plant protection chemicals, nutrients, and pharmaceuticals. So how should benefit be weighed given potential risks? Do the risks associated with transgenic approaches for metabolic trait improvements exceed that for conventional breeding practices? Is current metabolic profiling technology adequate for a targeted assessment of intended biochemical consequences? This review focuses on these issues by describing our current understanding of the possibilities of metabolic engineering through specific examples and with some emphasis on applications of RNAi technologies (Wesley et al. 2001; Watson et al. 2005; Tang et al. 2007). This review addresses the contribution of targeted metabolic profiling and biochemical theory for the development of new crops. The overall conclusion is that, as a class, novel crops including GM and non-GM crops should not automatically be considered safe, and that, consistent with numerous previous reviews and recommendations, case-by-case evaluations of new crops are more appropriate.

2 Overview of metabolic engineering

Metabolic engineering seeks to modify the amounts or chemical structures of specific metabolites through changes in the levels or activities of biosynthetic or catabolic enzymes, or the introduction of novel enzyme activities or regulatory proteins responsible for co-ordinate expression. The opportunities and challenges in metabolic engineering of secondary metabolic pathways have been reviewed (see for example Facchini et al. 2000; Hughes and Shanks 2002; Kutchan 2005; Sato et al. 2001; Trethewey 2004; Verpoorte and Memelink 2002).

Of course, one must address the question of how practical it is to predict which steps of a complex metabolic pathway need to be modified to successfully incorporate desired phenotypic effects. Metabolic Control Analysis (MCA) is a modeling device for complex biochemical systems which assumes control over flux is shared and dynamic among many enzymes. MCA aims to predict the most responsive steps to perturb in order to achieve desired metabolic modifications (Cornish-Bowden 1995; Kacser and Burns 1973; Rees and Hill 1994). Amongst other things MCA, as it is currently formulated, assumes the system is close to steady state and that the enzymes under investigation act in only one reaction. The complexities of whole plant systems have so far precluded MCA guiding attempts at secondary metabolic engineering in plants (Hughes and Shanks 2002) and it has not proven easy to predict the steps in a pathway whose modification is most likely to influence product accumulation. Confounding reasons for this include; (i) biosynthetic intermediates may be transported across a number of subcellular compartments (De Luca and St.Pierre 2000) and between cell types (Bird et al. 2003; Murata and De Luca 2005; Weid et al. 2004), (ii) intermediates may be handled by inter-dependent multi-enzyme complexes (*metabolons*) (Burbulis and Winkel-Shirley 1999), (iii) there may be competition between alternative biosynthetic pathways (Liu et al. 2002), and (iv) enzyme and pathway activities vary with time and developmental stage.

Given the complexity of intracellular and extracellular compartmentalization, it seems likely there will be a multiplicity of constraints on flux, including enzymatic, structural and transport limitations. Predictive methods will therefore remain elusive whilst empirical "trial-and error" methods will remain mainstream. Indeed, since transformation of most crops is relatively reproducible, albeit laborious, RNAi gene suppression is robust and accessible, and transgene expression is easily achieved, a wide range of test crop varieties with specific perturbations within a given metabolic pathway can be generated. Additionally, the metabolic profiling options, particularly through mass spectrometry (MS) and MS/MS, render it relatively straightforward to discover the most influential step in a target pathway. Thus, for each step where a gene is available, transgenic perturbations can be introduced to investigate the control points and bottlenecks of synthesis and accumulation. In other words, the current technologies for rapidly creating transgenic-based metabolically engineered crop varieties and for assessing the metabolic consequences in these new plants mitigate any current limitations in computationally-based predictive analyses.

The potential of transgenic-based metabolic engineering, and particularly the use of RNAi technology (Tang et al. 2007) in trait improvement, is now illustrated in the following examples.

3 Metabolic engineering of alkaloids in opium poppy

Papaver somniferum, oilseed or opium poppy, is one of the oldest cultivated plants and its analgesic use has a long history. The alkaloids in opium, the dried latex, are presumably involved in defense against microbial and animal predation. Codeine and morphine are generally the most abundant alkaloids in poppy, although induced mutants and natural variants can accumulate significant amounts of alternative intermediates such as oripavine and thebaine (Millgate et al. 2004). Codeine and morphine remain two of the most important and effective analgesics used in medicine worldwide. The cultivation of poppy continues to be the only commercial means to the production of morphinan opiates because de novo synthesis is uneconomic largely as a consequence of a structure with five centers of chirality. Semi-synthetic manufacture of other drugs uses poppy morphinans as feedstock. Examples include analgesics buprenorphine and oxycodone, both synthesized from thebaine.

Transgenic research in poppy over the last few years provides exciting examples that illustrate the prospects for metabolic engineering of complex plant secondary products. Examples summarized below demonstrate modifications achieved through transgenic expression and RNAi silencing of codeinone reductase activity.

Larkin et al. (2007) produced transgenic poppy with constitutively expressed cDNA of codeinone reductase (COR), the enzyme which catalyzes the penultimate step in morphine synthesis (Fig. 1). The transformed line was a commercial variety characterized by high morphine yields. The promoter was from the subterranean clover stunt virus segment 4, a constitutive promoter in plants (Schunmann et al. 2004a, b), and leaves from the transgenic species had approximately ten fold increased levels of *Cor* transcript compared to non-transgenic controls.

Most transgenic lines carrying the enhanced COR trait showed significant increases in capsule alkaloid content in

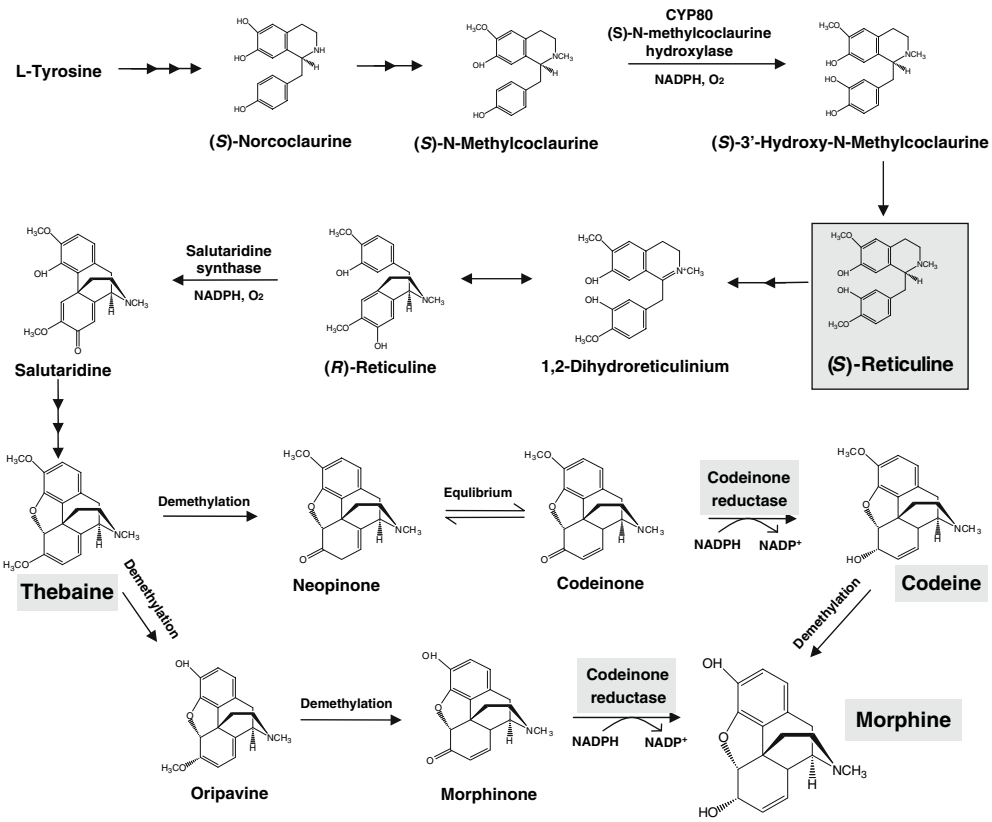

Fig. 1 Codeinone Reductase (COR) is the penultimate step in morphine synthesis. Transgenic over-expression of COR in the capsule results in the unexpected increase in morphine and codeine as predicted but also an unexplained increase in thebaine. Over-expression of COR leads to increases in morphine, codeine and thebaine. Silencing of COR expression in he capsule resulted in a surprising increase in (S)-reticuline which is seven enzymatic steps upstream of codeinone

replicated greenhouse and field trials conducted over a period of four years. Increases in morphinan alkaloid content were between 15% and 30% over the control genotypes and non-transgenic segregants. Two cycles of crossing of the best transgenic line were conducted into a more recent elite high morphine genotype. Backcross derivatives had significant increases in the levels of morphine and total alkaloids over the elite recurrent parent. As evaluated by HPLC-MS based metabolic profiling, the increase in total alkaloid content observed was usually attributable to increases in the levels of morphine, codeine and thebaine. Increases in morphine and codeine can be expected from an increase in COR expression. However the increase in thebaine represents a totally unexpected aspect of this study. Thebaine is an intermediate upstream of COR and we can only speculate on how its increased production was induced; the details and regulation of the cellular and subcellular localization of the various steps of the morphinan pathway remain largely unknown. We do know that various branches of benzylisoquinoline alkaloid biosynthesis are localized to vesicles in distinct cell types, the laticifers (Bock et al. 2002); and the early steps of the specific morphine branch appear to occur in different cell types to the later steps (Bird et al. 2003; Weid et al. 2004; Samanani et al. 2006). We might speculate that the *Cor* over-expression has induced a feed-forward activation of earlier steps and the increased accumulation of thebaine is a consequence of it being a final intermediate before transition to a separate compartment.

Nevertheless, these experiments illustrate the prospects for yield increases in complex pathways through simplistic modified expression of pathway enzyme genes. From the perspective of potential unintended consequences it also raises the question of whether increases in alkaloids might be occurring in off-target tissues with unwanted ecological effects. Fortunately, metabolic profiling can provide an insight into unintended consequences by assessing levels of all alkaloids present in a pathway (Allen et al. 2004; Raith et al. 2003) and is equally applicable to all tissues. Indeed, capabilities in MS-based profiling of the benzylisoquinoline pathway have now been expanded to stable isotope labeling studies by FT-ICR-MS (Schmidt et al. 2007)

In the COR expression study described above HPLC analyses of tissues other than capsules indicated that there are no major changes in alkaloid types or amounts across tissues that might be expected to have an ecological impact (Larkin et al. 2007). Although the introduced gene was driven by a constitutively expressing promoter, only codeine was significantly increased in tissues other than capsules and that occurred only in the lower and upper stem. The normal tissue distribution of alkaloids is likely preserved by the fact that all other genes of the pathway are

unchanged. This result is of practical significance in considering the likelihood of unanticipated ecological effects. A significant change of alkaloid type or quantity in roots might affect soil biota. Similarly, changes in pollen alkaloids might conceivably affect the behavior of bees or other pollinators. No changes were observed that would cause any suspicion of unintended consequences.

In contrast to transgenically modified expression, Allen et al. (2004) examined the consequences of silencing COR expression using a transgene designed to produce a chimeric hairpin RNA construct which would induce RNAi suppression of all known members of this multigene family. This required conserved sequences from two members of the family to be used in the transgene. Once again, metabolic profiling highlighted unexpected changes in benzylisoquinoline metabolism. The biosynthetic precursor (*S*)-reticuline (Fig. 1), as well as methylated derivatives of (*S*)-reticuline, codamine, laudanine and laudanosine, accumulated in transgenic plants with corresponding decreases in the levels of morphine, codeine, oripavine and thebaine. The accumulation of (*S*)-reticuline, seven enzymatic steps upstream of codeinone synthesis, was unexpected and indicates a feedback mechanism preventing intermediates from general benzylisoquinoline synthesis entering the morphine-specific branch. Analysis verified loss of *Cor* gene transcript, appearance of 22-mer degradation products and reduction of enzyme activity. However transcript levels for seven other enzymes in the pathway, both before and after (*S*)-reticuline were unaffected. A possible explanation for the observed long-range effect is that COR is part of a multienzyme complex (metabolon) which also involves the enzyme which acts on (*S*)-reticuline, namely 1,2-dehydroreticulinium ion synthase. Loss of COR would also suppress the activity of other enzymes of the complex. Obviously the enzymes of the proposed metabolon would have to be acting within the same compartment and the metabolon hypothesis remains to be tested. Again, the targeted use of metabolic profiling enabled the unpredicted changes in this pathway to be identified and characterized.

Other examples are emerging from the CSIRO morphine research in which transgenically modified expression of some but not all tested pathway genes results in increases in morphinan alkaloids, while RNAi silencing causes the accumulation of both expected and unexpected intermediates. These snapshots of ongoing benzylisoquinoline metabolism research highlight the value of transgenic approaches to modifying alkaloid content in opium poppy and particularly the use of RNAi silencing in effecting loss-of-function perturbations of metabolism. Metabolic profiling, particularly HPLC-MS based approaches, has been extensively applied in these studies to assess for unintended consequences in the targeted pathway and has proven particularly effective in this manner.

In this journal issue, Tang et al. (2007) emphasise that RNAi silencing is a natural mechanism and that transgenic DNA-encoded RNAi silencing mimics an endogenous process. So, should assessments of unintended consequences only be applied to crops modified transgenically or also to crops modified through conventional breeding? Or is it the modified trait that should be considered most relevant? In the case of poppy being developed strictly to yield purified pharmaceutical precursors, an extremely low level of risk will apply. A different level of risk may be considered for foodstuffs composed of whole plant tissues and intended for consumption. Consider the following two examples.

4 Metabolic engineering of lysine production in maize

Although extensively used as livestock feed, the nutritional value of maize grain is compromised by low levels of essential amino acids such as lysine and tryptophan; exogenous lysine, for example is typically required as a dietary supplement for animals such as swine and poultry. Two major strategies have been pursued to increase endogenous levels of lysine in grain; these include altering protein composition by modifying the levels of seed storage proteins or increasing levels of free lysine by modifying anabolic and catabolic steps involved in lysine biosynthesis.

Protein composition in maize grain can be dramatically impacted by a mutation in the *opaque 2* gene which encodes a b-ZIP transcription factor that controls expression of the lysine-poor zein storage protein (Azevedo et al. 2004). The opaque-2 mutation was pivotal to the development of Quality Protein Maize (QPM), the winner of the World Food Prize in 2000 (Prasanna et al. 2001; Vietmeyer 2000). However, whilst down-regulation of the expression of zein storage proteins results in an increase in lysine composition (through a concomitant increase in lysine-rich non-zein storage proteins) the opaque 2 mutation is typically associated with adverse effects on other agronomic traits including yield and kernel characteristics. The use of modifier genes is typically required for high quality QPM hybrids. RNAi silencing (Segal et al. 2003) and antisense (Huang et al. 2004, 2005) technologies have been pursued as alternative approaches to down-regulating zein expression without interfering with the general functions of the opaque-2 transcription factor. As with the previously discussed studies on opium poppy this research highlighted both the value of transgenic approaches in effecting loss-of-function perturbations in a highly specific manner and the use of profiling analyses of target metabolites (amino acids) to assess for intended and unintended compositional effects. Huang et al. (2005) demonstrated that targeted zein

protein reduction did not affect expression of the bifunctional lysine-ketoglutarate reductase/saccharopine dehydrogenase (LKR/SDH), a key enzyme in lysine catabolism (Fig. 2), whereas opaque-2 mutations typically have reduced activity (Gaziola et al. 1999). This reduction in LKR/SDH activity in opaque 2 mutants, along with other perturbations in the aspartate family metabolic network, can be further modulated by opaque 2 modifier genes (Gaziola et al. 1999). Reductions in LKR/SDH activity are also associated with increased levels of free lysine (Stepansky et al. 2005) and this enzyme may prove a potential target for increasing the nutritional value of maize grain. However, increases in free lysine levels in maize induced through pathway modification have, to date, been associated with safe but unintended increases in the metabolite

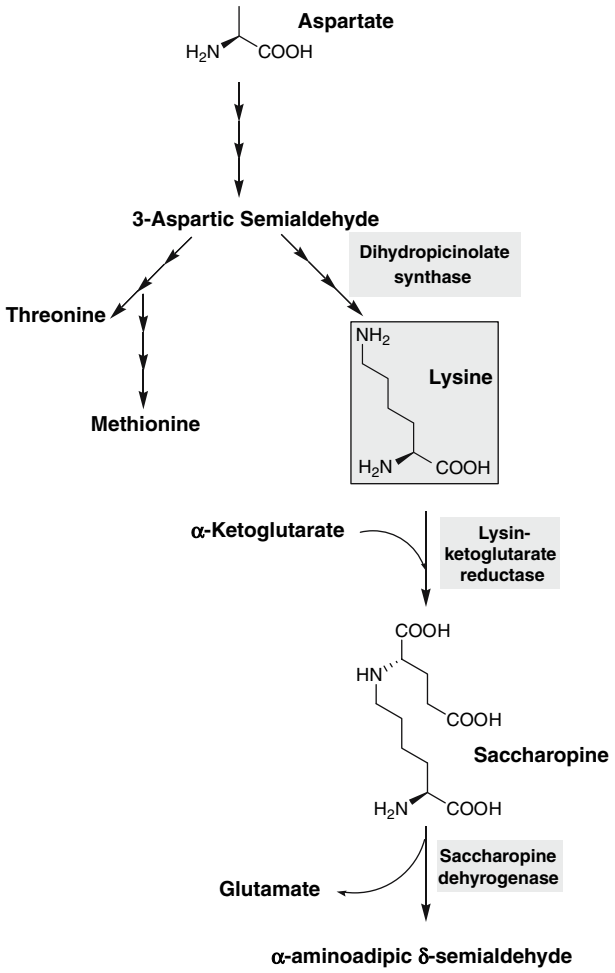

Fig. 2 Overview of lysine biosynthesis and catabolism. Over expression of a feedback insensitive dihydropicinolate synthase (DHPS) can elevate free levels of lysine in maize grain. Suppression of lysine catabolism through silencing of the bifunctional lysine-ketoglutarate reductase/saccharopine dehydrogenase (LKR/SDH) enzyme can also elevate lysine levels. Free lysine levels can be increased in opaque 2 mutations as an unintended effect

saccharopine (Huang et al. 2005). Perhaps of more relevance to the issue of unintended effects, however, is the fact that the interaction of free amino acid metabolic networks with protein composition (total amino acid content) is only partially understood and remains an active area of research (*e.g.* Galili et al. 2006). The opaque 2 mutation is also associated with increases in levels of numerous free amino acids, including free lysine in the mature maize grain (Wang and Larkins 2001). In other words, modification of zein content (the intended trait) is associated with unintended effects in free amino acid metabolism. The extent of this unintended effect appears to be germplasm dependent and several factors such as transport, increased amino acid synthesis, or reduced rates of amino acid incorporation into protein can, at least in principle, contribute (Wang and Larkins 2001). Of course, one can come to the realization that the term "unintended" is quite malleable and contingent on current biochemical knowledge but it is quite reasonable to suggest that modification of protein composition in plants will most certainly have unintended (but, quite often, anticipated) effects on free amino acid metabolism *regardless* of the means of trait incorporation.

Deregulating metabolic pathways associated with lysine biosynthesis as well as lysine catabolism (Fig. 2) can also yield high-lysine maize varieties. The first committed step of lysine biosynthesis is catalyzed by dihydropicolinate synthase (DHPS) which is normally highly sensitive to feedback inhibition by lysine. Transgenic expression of the *Cornyebacterium glutamicum* gene *cordapA* which encodes a feedback-insensitive DHPS protein allows lysine biosynthesis to continue even in the presence of high levels of free lysine (Huang et al. 2005). Indeed, this approach has led to the commercial development of high-lysine maize that does not require lysine supplementation when used as a feedstock. Metabolic profiling of free amino acids in a high lysine maize line revealed reductions in the contents of aspartate and glutamate as were fully predicted, but also elevation in saccharopine content (Huang et al. 2005). This elevation is unintended yet reasonable and not unanticipated.

As mentioned earlier, inhibition of lysine catabolism through suppression of LKR/SDH activity can further increase levels of free lysine (Stepansky et al. 2005). As also pointed out earlier, reduction in the activity of this enzyme is now recognized as a pleiotropic effect in opaque-2 mutants. This results in an interesting situation in which transgenic crops potentially modified to silence LKR/SDH expression may require a full safety assessment whereas conventional crops bred for increased lysine content need undergo no such safety assessment nor any evaluation of pleiotropic effects on enzymes associated with lysine metabolism. It should be apparent that, in many regards,

the effects of transgenic modification can be addressed more specifically than the effects of traditional breeding. Furthermore, metabolic profiling of free amino acids and of lysine catabolites such as saccharopine and α-amino-adipic acid is now a well established technology and applicable to modification of lysine traits regardless of the means of modification. This brings us once again to the quote by Cellini et al. (2004) "Unintended effects occur in both GM and non-GM crops; however, GM crops are better characterized. It may be suggested that the two should be treated the same in safety assessments, bearing in mind that safety assessments are not required for non-GM crops".

5 Metabolic engineering of gossypol production in cotton

As a crop, the major utility of cotton is fiber production. Transgenic modification of cotton has centered primarily on incorporating insecticidal activity (Perlak et al. 2001) or herbicidal resistance (Nida et al. 1996) as a means of ensuring robust and high-yielding fiber production. Cottonseed is used as an animal feed. Yet cotton may have additional value as a foodstuff in developing countries where malnutrition and starvation are widespread. In principle, the cottonseeds that remain after fiber extraction offer a potential source of nutrition; for every single pound of fiber produced through cotton, over 1.6 pounds of protein-rich cottonseed is produced. Cottonseed however contains the toxic (and male contraceptive) gossypol (Fig. 3). Gossypol and other related terpenoids are also produced in vegetative and reproductive tissue where it confers protection from insects and other pathogens. Conventional breeding to remove gossypol from cottonseed has been pursued for over fifty years. So-called "glandless" cotton lacking the gland that produces gossypol has been generated but the lack of tissue-specificity has rendered these cultivars highly susceptible to insect predation (Lukefahr et al. 1966). As such, they have no commercial utility and cottonseed-specific suppression of gossypol has remained a long-standing goal. Recently however, transgenic cotton plants expressing an RNAi construct of the δ-cadinene synthase gene required for gossypol synthesis fused to a seed-specific promoter have been generated. These plants are associated with seed-specific reduction of gossypol, along with the biosynthetically related heliocides, whilst levels in non-seed tissues are comparable to that in control plants (Sunilkumar et al. 2006). These cotton plants are thus expected to have similar insect and pathogen resistance to that of wild type cotton, but to produce seeds with high nutritional value. The importance of RNAi technology, and its relevance to "detoxifying" other potential foodstuffs such as *Lathyrus sativus*, is becoming very evident.

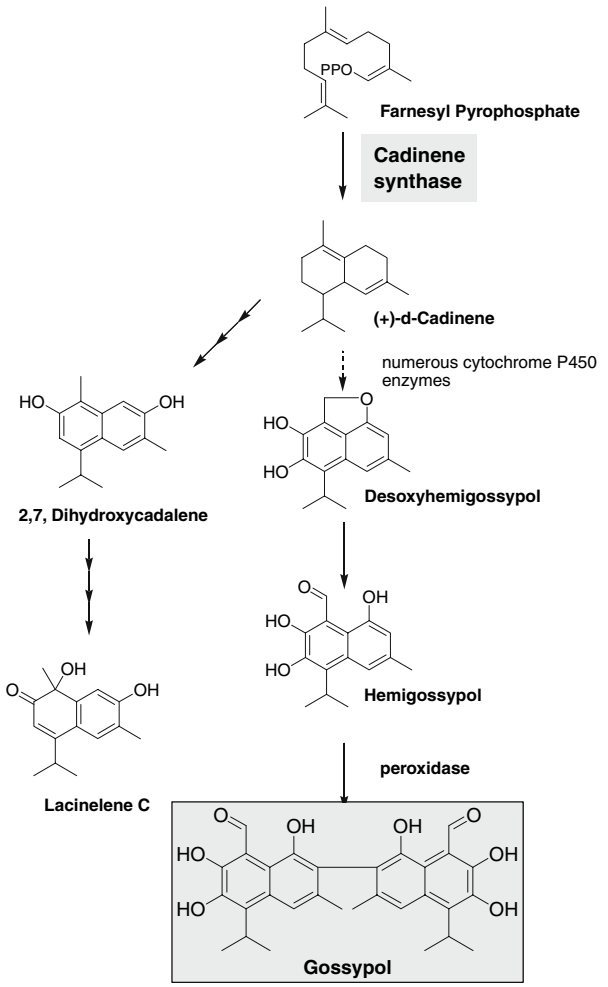

Fig. 3 Proposed biosynthetic pathway for gossypol from farnesyl pyrophosphate. RNAi silencing of cadinene synthase results in suppression of gossypol production in cottonseed

We have implied throughout this article that metabolic profiling and compositional analyses play an important role in any trait development involving metabolic engineering. Two issues of direct relevance are quite apparent. Firstly, development of the gossypol-deficient cotton cultivars was supported by profiling of gossypol and related intermediates such as the heliocides (see Benson et al. 2001 for example of methodology). Secondly development and regulatory oversight of GM cotton has led to a greater understanding of natural variation in the composition of cottonseed and cottonseed oil (Bertrand et al. 2005; OECD 2004). The ILSI crop composition database (Ridley et al. 2004) currently maintains compositional data on approximately 215 seed samples harvested from a diverse range of non-GM crops that can be used as a basis for comparison to GM crops. This includes data on other anti-nutrients present in cottonseed such as the cyclopropanoid fatty acids (Wood et al. 1994). This type of information will clearly be valuable in assessing

for unintended effects in any new cottonseed varieties intended for human consumption.

It is also relevant that the comparative safety assessment process, in many countries, calls for comparison of the test GM crop against a conventional counterpart with a demonstrated "history of safe use". This can be considered to preclude cottonseed because it has no history as a food, although cottonseed oil does have applications here. As such, it is obvious that compositional and metabolic profiling technologies, in conjunction with animal studies, will have an important role here in both safety assessments and in assessing the efficacy of RNAi approaches to gossypol reduction in cotton and, indeed, more generally to the use of RNAi silencing to "detoxify" other potential foodstuffs.

The above three examples highlight the versatility and potential of metabolic engineering through transgenic modification. The discussion on poppy benzylisoquinoline alkaloids and on maize lysine metabolism also emphasize that surprises - unintended consequences - can occur and that this is true of even conventional crops such as the opaque 2 maize mutants. Options for more discrete and targeted metabolic modifications are greatly enhanced by the transgenic approach, however. Of course, we do not know, as yet, to which extent unintended consequences, specifically changes in other anti-nutrients will be associated with gossypol-deficient cottonseed. It is most likely however that current compositional analyses and new developments in metabolic profiling can inform requirements for such assessments.

Critics of the introduction of GM crops often refer to some form of *precautionary principle*. This can sound very reasonable for making policy judgments on the introduction of a new technology or entity: do not introduce it if there are potential risks unless and until it is proven safe (see Van den Belt 2003). However we can only understand new risks by comparison with risks that we already know and work with—risks are relative. It is not possible to turn the precautionary principle into a quantifiable basis of action. More importantly, it is extremely easy to turn the precautionary principle into a means to impede progress in any arena. One can easily use the precautionary principle to mount a compelling case against organic food and herbal remedies. With respect to GM technology, a more appropriate approach to risk assessment is to place all potential risks that can be rationally imagined for a GM crop variety against analogous risks that we readily live with.

There are risks of unintended consequences associated with both conventional and transgenic modes of plant breeding. We must ask: do surprising metabolic consequences in transgenics constitute unacceptable risk? Do differences in the levels of free amino acids, for example, really constitute a safety concern? Do differences we observe between GM crops and their conventional counterparts exceed differences attributable to natural variation in conventional ecotypes grown at distinct geographical regions? We can also ask: does our reluctance to determine whether surprising metabolic consequences occur in conventionally bred crops constitute unacceptable risk? Should the potential for risk be weighed against the potential for benefit, a question of considerable relevance to the potential development of gossypol-free cottonseed? Answers to such questions ultimately require a realistic appraisal of the nature and genetic plasticity of the crops we consume daily.

In reality plants can be dangerous and foods can kill. Some people are dangerously allergic to otherwise safe and nutritious foods such as peanuts, kiwi fruit or shellfish. The precautionary principle would ban them if applied consistently. We live with those risks without too much alarm or excessive regulation. Plants are genetically programmed to synthesize a large battery of poisons and irritants, so called secondary metabolites, designed to ward off microbes, insects and animals from eating them. Over a long period mankind has selected and bred varieties of domesticated foods where the "nasty" genetics and chemistry have been minimized for our convenience and survival. However the poisonous chemical capacity is not altogether eliminated. Conventional/natural varieties of our foods have variable gene expression. Every time a cross is made genetic rearrangements are possible; genes that were previously silent can be activated and vice versa. Sometimes these changes can result in poisoning; there are documented examples in new conventional varieties of celery, squash, zucchini and potato where poisoning, skin rashes and even deaths have occurred (Akeley et al. 1968; Rymal et al. 1984; Zitnak and Johnson 1970; Seligman et al. 1987; Vaananen et al. 2005; Cellini et al. 2004).

Neither does it appear to be widely acknowledged that genetic mutability is occurring in our food crops at a high level and by a number of mechanisms only some of which we understand. Transposons or so-called *jumping genes* may be active; every generation in the life of a crop variety they have opportunity to move; and every time they move they can turn some silenced genes on and turn other genes off. We can sometimes observe their activity if they are landing in or jumping out of genes which control colors. The color patterning in many flowers is an attractive example of such gene mutability. For every visible manifestation of a jumping gene we can expect there to be over a thousand invisible jumps. We are happy to eat an apple with spotty red patterns on the skin. Yet those patterns are a reminder that genes may have been switched on for the production of a toxic chemical. An apple under environmental stress, such as extra pest predation in an *organic* orchard, is even more likely to have jumping gene mutations. Stresses such as hydrostatic pressure have been shown to induce a wave of transposon activity in rice

(Long et al. 2006). The introduction of a fragment of chromosome from a sexually compatible species can also trigger transposon activity (Shan et al. 2005). These types of genetic changes can be happening subsequent to the release of a variety, so that scrutiny prior to release cannot entirely guard against a dangerous change.

Transposons are not the only mechanism for unintended genetic changes in conventional breeding. Consider the murky but natural world of epigenetics. One of the mechanisms a plant uses to control gene activity is DNA methylation. This methylation pattern represents a layer of information over and above the basic information in the gene and can dramatically alter the level of gene expression. Recent studies in rice have revealed extensive change in DNA methylation (as well as gene copy number and sequence) following a conventional breeding program involving the introduction of a chromosomal translocation of a sexually compatible species (Liu et al. 2004; Long et al. 2006; Chen et al. 2006). These and other natural mechanisms of gene mutability are at play in your food—whether GM or non-GM. Notably, a recent global study of all expressed genes, transcriptome, in leaf and endosperm in wheat demonstrated that the differences in gene expression were much greater between two conventionally bred varieties than between a transgenic and recipient wheat (Baudo et al. 2006).

So, genetic mutability is a natural and frequent risk in plant breeding and food production; these are risks we live with now. The risks associated with a GM variety are not inherently less, but are much less in practice because of the high level of genetic and food safety assessment required of it. Yet, a gene incorporated into a GM plant may knock out another gene or it may lose activity over time. This represents uncertainty. There are examples in the laboratory of exactly this happening. One of the reasons for extensive analysis of transgenic events is to find specific transgenic lines which have demonstrated no secondary effects and which prove stable in their expression over many generations. Regulations worldwide require the place of integration of the transgene to be defined and understood. Therefore the risk of unintended consequence, although real, is greatly minimized by analysis and screening of the specific transgenic events proposed for commercialization. This level and scope of testing of a potential new GM variety is orders of magnitude greater than required for conventionally bred varieties and this is associated with a higher level of security and safety.

6 Concluding remarks

As exemplified in our earlier discussion on metabolic engineering it is clear that unintended genetic, metabolic and compositional consequences are possible from any form of genetic modification, including traditional breeding and transgenic modification. In a medicinal crop like poppy, there were unanticipated effects generated from metabolic modifications of the benzylisoquinoline pathway. Of course in a medicinal crop risks are reduced as the products are sold as purified pharmaceuticals. For a food crop the potential for unintended consequences extends to the entire chemical components of one or more plant tissue. Clearly the number of metabolites present is very large and the growing power of targeted metabolic profiling will become increasingly useful in analyzing the chemical complexity of prospective commercial releases as they progress through initial research and development phases. As we noted earlier plants can produce a vast armory of toxic substances. The risks of unintended metabolic changes are real from any form of breeding and potentially even in crops designed to reduce toxic substances such as gossypol in cotton. However, genetically engineered plants may represent much less risk to the public because not only do they undergo a high degree of scientific scrutiny during the risk assessment phase but must survive an extensive screening process prior to selection as a prospective commercial release. The methodologies involved in these screening processes will continue to improve with new developments in targeted metabolic profiling. The ability to detect potential unanticipated effects during the process will greatly inform compositional requirements for safety assessments.

An unbiased assessment of the safety of GM food crops thus far released after they have passed the multifaceted scrutiny to which they are subjected by law in their various jurisdictions, would have to conclude that they are extremely safe. Because of the regulatory requirements placed on those wishing to release a new GM crop variety, most of the safety assessment studies have been performed or commissioned by the organizations or companies wishing to release. One review publish in 2004 (Preston 2004) highlighted that at that time 42 peer-reviewed original research studies had been published in which GM crop products have been fed to mammals, birds or fish and compared to non-GM feed (Preston 2004): (i) 36 of these studies showed no difference; (ii) four showed better outcomes from GM (but 2 of these were GM modifications intended to be beneficial as food); and (iii) two early studies showed negative effects on rodents, but these (Ewen and Pusztai 1999) involved analysis of a transgenic product (snowdrop lectin) suspected (but not proven) to be toxic and not intended for commercial release (and even this study is suspect due to methodological criticism including lack of proper controls (see Enserink 1999 and Fedoroff 2006). The number of peer-reviewed studies in which GM crops have been used in feeding studies now numbers over 100 and all consistently show no significant differences in outcomes when compared to the effects

non-transgenic crops (Flachowsky et al. 2005; Flachowsky et al. 2007).

In summary, our overview of diverse metabolic engineering applications in a range of plants with diverse uses (potential and otherwise) has highlighted that unintended consequences are real but that modern targeted metabolic profiling technologies can rapidly identify pathway perturbations. If incorporated into the early selection stages of a prospective new trait targeted metabolic profiling may greatly aid in the selection of metabolites that need to be considered during the compositional phase of a risk assessment. These observations can obviously be extrapolated to the "GM universe" to suggest that compositionally based safety assessments can contribute to the security of our food chain. However, it should be self-evident that GM crops ought not to be considered a single monolithic class that is either good or bad for the economy, agriculture or the environment. Each novel crop should be considered on its own merits and demerits. If we ever get to that point we will have achieved something positive out of the GM controversy.

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