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ORIGINAL ARTICLE

Lessons learned from metabolic engineering of cyanogenic glucosides

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Abstract Plants produce a plethora of secondary metabolites which constitute a wealth of potential pharmaceuticals, pro-vitamins, flavours, fragrances, colorants and toxins as well as a source of natural pesticides. Many of these valuable compounds are only synthesized in exotic plant species or in concentrations too low to facilitate commercialization. In some cases their presence constitutes a health hazard and renders the crops unsuitable for consumption. Metabolic engineering is a powerful tool to alter and ameliorate the secondary metabolite composition of crop plants and gain new desired traits. The interplay of a multitude of biosynthetic pathways and the possibility of metabolic cross-talk combined with an incomplete understanding of the regulation of these pathways, explain why metabolic engineering of plant secondary metabolism is still in its infancy and subject to much trial and error. Cyanogenic glucosides are ancient defense compounds that release toxic HCN upon tissue disruption caused e.g. by chewing insects. The committed steps of the cyanogenic glucoside biosynthetic pathway are encoded by three genes. This unique genetic simplicity and the availability of the corresponding cDNAs have given cyanogenic glucosides pioneering status in metabolic engineering of plant secondary metabolism. In this review, lessons learned from metabolic engineering of cyanogenic glucosides in *Arabidopsis thaliana* (thale cress), *Nicotiana tabacum* cv Xanthi (tobacco), *Manihot esculenta* Crantz (cassava) and *Lotus japonicus* (bird's foot trefoil) are presented. The importance of metabolic channelling of toxic intermediates as mediated by metabolon formation in avoiding unintended metabolic cross-talk and unwanted pleiotropic effects is emphasized. Likewise, the potential of metabolic engineering of plant secondary metabolism as a tool to elucidate, for example, the impact of secondary metabolites on plant–insect interactions is demonstrated.

Keywords Cyanogenic glucosides · Metabolic engineering · RNAi · Transgene silencing · 5′-Azacytidine

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1 Introduction

Genetic engineering provides a valuable approach to alter and improve metabolite composition in crop plants to generate robust plants with improved traits of interest to consumers and producers. Plants can be genetically engineered to have higher nutritional value or tailor-made resistance against pathogens and herbivores. Genetic engineering also holds promise for economically favourable production platforms for bio-pharmaceuticals, essential oils, colorants, flavours and fragrances. Examples of genetic modification of crops to improve nutritional quality include

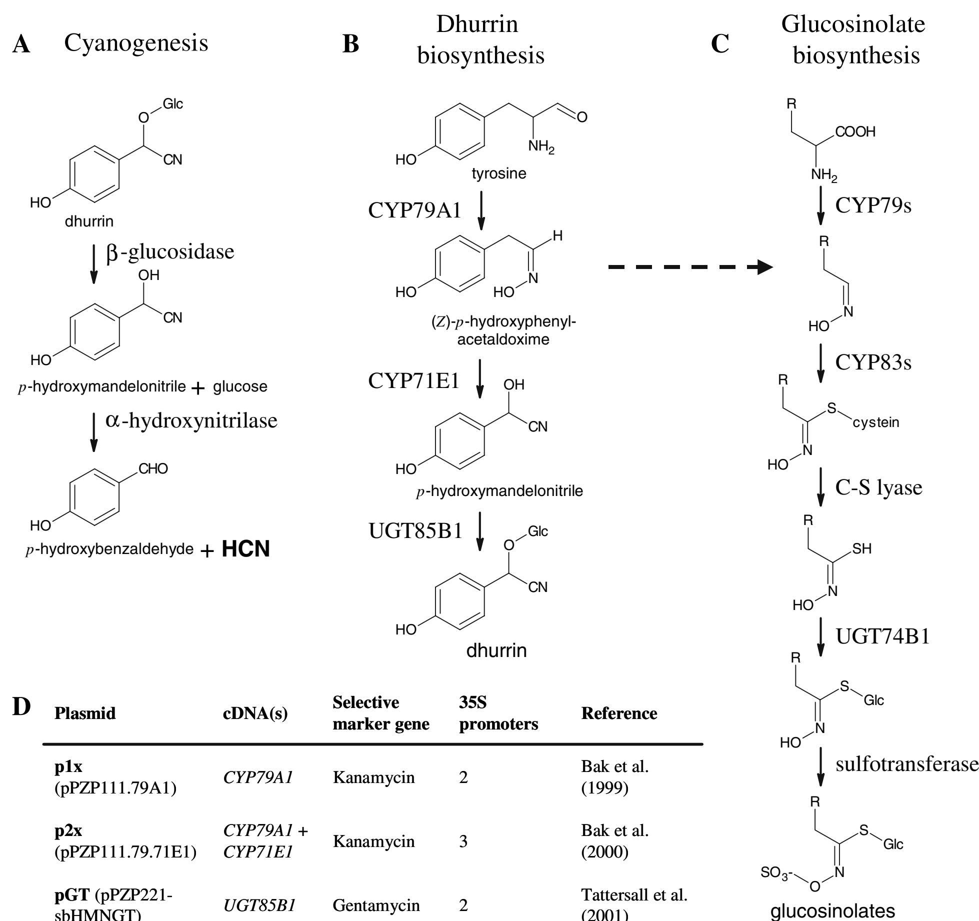
"Golden Rice" with increased pro-vitamin A content (Ye et al. 2000), oil crops accumulating essential very long chain polyunsaturated fatty acids (Abbadi et al. 2004; Wu et al. 2005), potatoes that accumulate storage proteins with increased levels of essential amino acids (Chakraborty et al. 2000), iron and zinc enriched rice (Vasconcelos et al. 2003) and low allergen soybean (Herman et al. 2003). Plant metabolism is highly complex, and predictive metabolic engineering is often hampered by a lack of detailed knowledge about metabolic cross-talk and regulation of metabolic grids. A major challenge in metabolic engineering is to design and construct plants with a limited number of unintended side effects and to reduce the number of unexpected results by enhancing our ability to carry out *in silico* prediction of metabolic responses to alterations in biosynthetic pathways.

Of particular interest is the ability to engineer plants with new or altered levels of secondary metabolites often referred to as natural products. Plants have the capacity to synthesize a vast range of secondary metabolites making plants the organic chemist par excellence in nature. The impact of secondary metabolites in the successful adaption of plant species cannot be underestimated as these highly sophisticated small metabolites have evolved during millions of years of selection during speciation. They are important players in adaptation to abiotic and biotic stresses such as acclimation and plant–insect and plant–microbe interactions, as they provide the chemical signals that enable plants to deter herbivores and pests, attract pollinators, communicate with other plants and constantly adapt to climatic changes. Through time, man has relied upon and exploited the use of plant secondary metabolites as flavours, scents, poisons, natural pesticides and pharmaceuticals. Not only are secondary metabolites the active components in traditional herbal medicines, they are often the origin and/or the precursor of most of today's medicine (Morant et al. 2003). The exploitation of plant secondary metabolites is often hampered by their accumulation in too low amounts or by the fact that their occurrence is restricted to a single or few exotic plants not suited for commercialization. In addition, a lack of knowledge of the enzymatic steps and, in particular, the unavailability of the underlying genes encoding the requisite enzymes present major limitation to the exploitation of plants as green factories for production of desired secondary metabolites (Kutchan 2005).

Cyanogenic glucosides constitute a limited number of amino acid derived secondary metabolites found throughout the plant kingdom (Bak et al. 2006). What makes cyanogenic glucosides interesting from a metabolic engineering point of view is that this group of compounds is thought to play an important role in the ability of plants to combat pests and diseases. In addition, the entire pathway has been elucidated and the cDNAs encoding the three enzymes that catalyze the committed steps have been isolated. The availability of the cDNAs and the unique genetic simplicity have given the cyanogenic glucoside pathway its pioneering status in metabolic engineering.

When the cellular structure of tissues that accumulate cyanogenic glucosides is disrupted, e.g. by a chewing insect, the cyanogenic glucosides are released from the vacuoles and hydrolyzed by specific β-glucosidases to yield glucose, a ketone or an aldehyde and toxic HCN. This process is known as cyanogenesis and serves to facilitate a rapid HCN release (Fig. 1A). Thus cyanogenic plants possess an immediate defense system against attacking herbivores. The cyanogenic glucoside pathway has been extensively studied in *Sorghum bicolor* (great millet) and it has been shown that the entire pathway is encoded by only three genes (Bak et al. 2006). The conversion of tyrosine into dhurrin in *S. bicolor* seedlings is catalyzed by the sequential action of two multifunctional cytochrome P450 enzymes, CYP79A1 and CYP71E1, and a soluble family 1 UDP glucosyl transferase, UGT85B1 (Fig. 1B; Bak et al. 1998a; Halkier et al. 1995; Jones et al. 1999). *S. bicolor* seedlings accumulate up to 30% dry weight of the tyrosine-derived cyanogenic glucoside dhurrin in the seedling tip while at later life stages the level of dhurrin is notably reduced (Busk and Møller 2002; Halkier and Møller 1989).

Transfer of the entire dhurrin pathway from *S. bicolor* into a distantly related plant species using genetic engineering has provided a powerful tool to study the impact of dhurrin as a defense compound (Tattersall et al. 2001), because this offers the possibility to eliminate the impact of natural variation with respect to metabolite composition and morphology which is otherwise often encountered when ecotypes and mutants are used to study plant–insect interactions. Expression of enzymes from the dhurrin pathway in *Arabidopsis thaliana* (thale cress) offers the opportunity to study metabolic cross-talk between the structurally related cyanogenic glucoside and glucosinolate pathways, as well as the impact of dhurrin and cyanogenesis on insects that specifically feed on cruciferous plants such as the cruciferous feeding specialist flea beetle *Phyllotreta nemorum* (Tattersall et al. 2001). *A. thaliana* is easy to transform and its entire genome has been sequenced which makes it an obvious choice for genetic engineering. As a cruciferous plant, *A. thaliana* does not synthesize cyanogenic glucosides but produces glucosinolates, a group of amino acid derived phytoanticipins related to, but not naturally co-occurring with, cyanogenic glucosides (Fig. 1C; Halkier and Gershenzon 2006). While cyanogenic glucosides are synthesized from tyrosine, phenylalanine, leucine, isoleucine, valine and 2-cyclopentenylglycine, glucosinolates are derived from methionine, alanine, isoleucine, valine, leucine, tryptophan, tyrosine and phenylalanine by a



Plasmid	cDNA(s)	Selective marker gene	35S promoters	Reference
p1x (pPZP111.79A1)	*CYP79A1*	Kanamycin	2	Bak et al. (1999)
p2x (pPZP111.79.71E1)	*CYP79A1* + *CYP71E1*	Kanamycin	3	Bak et al. (2000)
pGT (pPZP221-sbHMNGT)	*UGT85B1*	Gentamycin	2	Tattersall et al. (2001)

Fig. 1 Metabolism of dhurrin and glucosinolates. (**A**) Cyanogenic glucosides like dhurrin are stored in vacuoles separately from the catabolic *β*-glucosidases that serve to bioactivate the defense compounds to yield *p*-hydroxymandelonitrile which either enzymatically or spontaneously dissociates into an aldehyde and toxic HCN. (**B**) In *S. bicolor*, tyrosine is converted into dhurrin by the sequential action of two membrane bound cytochromes P450 and a family 1 UDP glucosyl transferase. The multifunctional CYP79A1 catalyzes the first committed step in dhurrin biosynthesis, the conversion of tyrosine into *p*-hydroxyphenylacetaldoxime. The oxime is subsequently converted into *p*-hydroxymandelonitrile by the action of CYP71E1. *p*-Hydroxymandelonitrile is immediately glucosylated by UGT85B1 into the cyanogenic glucoside dhurrin. (**C**) The two first committed steps of glucosinolate biosynthesis are similar to those of cyanogenic glucoside biosynthesis. Expression of *S. bicolor CYP79A1* in *A. thaliana* results in metabolic cross-talk at the oxime level as indicated by the *dotted arrow*. (**D**) List of transformation constructs applied to transfer the dhurrin pathway from *S. bicolor* to *A. thaliana* and *L. japonicus*

biosynthetic pathway whose initial reaction steps are equivalent to those of the cyanogenic glucoside pathway (Bak et al. 1998b). In both pathways, a multifunctional cytochrome P450 belonging to the CYP79 family catalyzes the conversion of the precursor amino acid or the chain elongated amino acid into the corresponding oxime which is further metabolized by a second cytochrome P450 belonging to either the CYP71 or CYP83 family (Fig. 1B, C; Bak et al. 2006; Halkier and Gershenzon 2006).

The leguminous plant *Lotus japonicus* (bird's foot trefoil) accumulates the aliphatic cyanogenic glucosides linamarin and lotaustralin derived from valine and isoleucine, respectively, and the isoleucine-derived cyanoalkenyl glucosides, rhodiocyanoside A and D (Forslund et al. 2004). Rhodiocyanosides constitute a class of glucosides that are related to the cyanogenic glucosides, but whose biological function is currently not understood. While rhodiocyanoside A and D are degraded by the same *β*-glucosidases that degrade the cyanogenic glucosides (our unpublished results), hydrolysis is not accompanied by release of HCN, as rhodiocyanosides are not derived from *α*-hydroxynitriles (cyanohydrins). While the ability to produce linamarin and lotaustralin is widespread in *Lotus* species, the ability to produce rhodiocyanosides appears to be limited to *L. japonicus* (Zagrobelny et al. 2007 and our unpublished results).

Flea beetles have co-evolved with cruciferous plants and have adapted to the presence of glucosinolates in their diet. In a similar manner, *Zygaena* species (burnet moths) have co-evolved with *Lotus* species. *Zygaena* larvae feed on

Fabaceous plants including *Lotus* species and sequester the cyanogenic glucosides for use in their own defense against predators. In the absence of sufficient amounts of cyanogenic glucosides in their dietary plants, the larvae possess the ability to de novo synthesize the very same cyanogenic glucosides, linamarin and lotaustralin, as present in *Lotus* species, albeit with a resultant concomitant reduction in growth rate (Zagrobelny et al. 2004). Metabolic engineering of *L. japonicus* to obtain plants with altered cyanogenic glucoside profiles, i.e. by introduction of novel cyanogenic glucosides such as dhurrin, or silencing of the endogenous cyanogenic glucoside pathway to obtain plants depleted of cyanogenic glucosides and rhodiocyanosides, thus provides a unique opportunity to elucidate the impact of cyanogenic glucosides on plant–insect interactions using the unique *Zygaena*—*Lotus* system.

Similar to *Lotus* species, the key stable crop *Manihot esculenta* Crantz (cassava) contains the cyanogenic glucosides linamarin and lotaustralin. *M. esculenta* is the world's most important tropical root crop and serves as a famine-reserve in the third world, especially in Africa (Nweke et al. 2002). A major nutritive drawback is the accumulation of up to 1.5 g/kg dry weight of linamarin and lotaustralin in the *M. esculenta* tubers (Bokanga 1994). Consequently it is of great interest to develop *M. esculenta* with acyanogenic tubers to provide a healthier diet for millions of people in the third world.

In this review, we report the lessons learned from studies on engineering the dhurrin biosynthetic pathway from *S. bicolor* into *A. thaliana* and *L. japonicus* and from silencing cyanogenic glucoside biosynthesis in *M. esculenta* and *L. japonicus*. We conclude that predictive metabolic engineering requires not only understanding of the metabolic pathways of the plant in question and the engineered pathway in particular, but also of transport and accumulation of the novel product as well as the ability of the plant to accommodate the transgenes and their encoded proteins.

1.1 Engineering metabolic cross-talk between the glucosinolate and cyanogenic glucoside pathways in *A. thaliana* changes the glucosinolate profile

The pathway for cyanogenic glucoside biosynthesis is wide spread and ancient (Bak et al. 2006). In contrast, the glucosinolate pathway is mainly present in Brassicales within eurosides II (Bak et al. 1998b; Halkier and Gershenzon 2006) and is generally thought to have evolved from a cyanogenic glucoside predisposition (Bak et al. 1998b). Accordingly, new metabolic pathways can be introduced into a plant species by combining parts of the two pathways by genetic engineering. This was first shown by expression of *S. bicolor CYP79A1* in *A. thaliana* that resulted in the production of high levels (up to 3% dry weight) *p*-hydroxybenzylglucosinolate, a tyrosine-derived glucosinolate not normally found in *A. thaliana* (Fig. 2, panel 1x). The introduction of *S. bicolor* CYP79A1 is manifested as a metabolic cross-talk that facilitates conversion of the otherwise toxic *p*-hydroxyphenylacetaldoxime to *p*-hydroxybenzylglucosinolate. Thus in these experiments, the glucosinolate pathway functions as a metabolic sink for the *p*-hydroxyphenylacetaldoxime generated by *S. bicolor* CYP79A1. While CYP79 enzymes in both the glucosinolate and cyanogenic glucoside pathways are notoriously known to exert a high degree of substrate specificity, the post oxime enzymes in the cyanogenic glucoside as well as the glucosinolate pathway are known to exhibit a rather broad specificity. This is a result of low specificity for the structure of the side chain of the substrate but high specificity for the presence of the functional group (Andersen et al. 2000; Bak et al. 2001; Bak and Feyereisen 2001; Forslund et al. 2004; Hansen et al. 2001; Mikkelsen and Halkier 2003; Naur et al. 2003). The ability to produce *p*-hydroxybenzylglucosinolate by expression of *S. bicolor CYP79A1* clearly demonstrates the flexibility of the post oxime metabolizing enzymes in the glucosinolate pathway. The transgenic *A. thaliana* 1x lines do not display any apparent visual phenotype as a result of the metabolic cross-talk generated with the glucosinolate pathway and the production of a new glucosinolate (Figs. 1B, C, 2, panel 3x).

The total level of glucosinolates in *A. thaliana* lines that express *S. bicolor CYP79A1* is four times higher compared to wild type, i.e. *p*-hydroxybenzylglucosinolate accounts for ~75% of the total amount of glucosinolates (Bak et al. 1999). In spite of this, two species of flea beetles, *P. nemorum* and *P. cruciferae* did not discriminate between *A. thaliana* 35S::*CYP79A1* and wild type in free choice feeding experiments (Nielsen et al. 2001). This demonstrates that neither a significant change in total glucosinolate content nor glucosinolate profile is important for the ability of flea beetles to recognize and feed on *A. thaliana*.

In a similar series of experiments, *S. bicolor CYP79A1* was expressed in transgenic *Nicotiana tabacum* cv Xanthi (tobacco) plants (Bak et al. 2000). The transgenic *N. tabacum* plants are reduced in height, produce a very limited number of seeds and accumulate metabolites derived from detoxification of *p*-hydroxyphenylacetaldoxime (our unpublished results).

Subsequent to the production of the transgenic *A. thaliana* plants producing a new tyrosine-derived glucosinolate, *A. thaliana* plants have been engineered to accumulate high amounts (up to 35% of total glucosinolate content in mature rosette leaves) of valine- and isoleucine-derived glucosinolates (Mikkelsen and Halkier 2003). This was achieved by expression of *M. esculenta CYP79D2*, the gene encoding

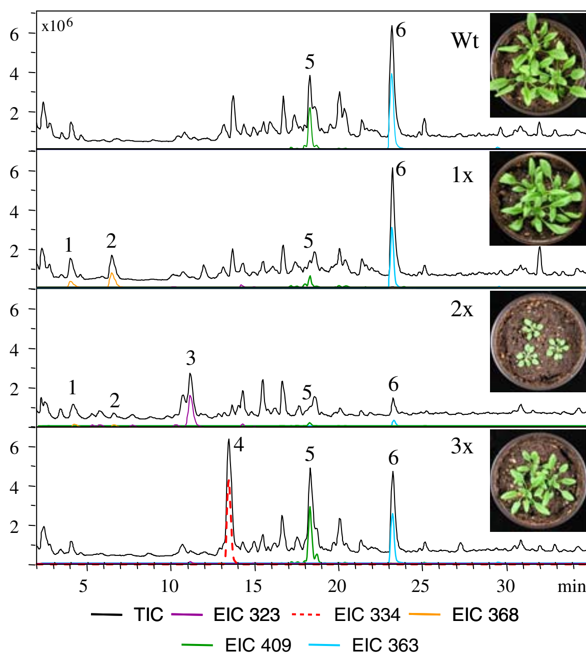


Fig. 2 Metabolite composition in *A. thaliana* wild type and transgenic lines. Plants transformed with *S. bicolor CYP79A1* are designated 1x. Plants transformed with *CYP79A1* and *CYP71E1* are designated 2x, and plants transformed with *CYP79A1*, *CYP71E1* and *UGT85B1* are designated 3x. The corresponding visual phenotypes of the transgenic plants are shown. Total ion chromatograms (TIC) and selected extracted ion chromatograms (EIC) for the sodium adducts of *p*-hydroxybenzylglucosinolate (1: EIC 368), desulfobenzylglucosinolate (2: EIC 368), *p*-hydroxybenzoylglucose (3: EIC 323), dhurrin (4: EIC 334), sinapoyl glucose (5: EIC 409) and sinapoyl malate (6: EIC 363) are shown. A more detailed analysis of the metabolite composition is presented in Kristensen et al. (2005) along with the methods used for analysis of metabolites

the enzyme that catalyzes the conversion of valine and isoleucine to the corresponding aldoximes in the cyanogenic glucoside pathway in *M. esculenta* (Andersen et al. 2000). These results confirm that the substrate specificity of the CYP79 enzymes is a major determinant of the glucosinolate profile and substantiate the broad substrate specificity of the post oxime enzymes in the glucosinolate pathway.

1.2 Metabolic engineering of dhurrin biosynthesis in *A. thaliana*: Efficient channelling of biosynthetic intermediates and resistance to flea beetles

A major break through in metabolic engineering of secondary metabolites in plants was the ability to introduce the cyanogenic glucoside dhurrin into *A. thaliana* plants with marginal impact on visual plant phenotype, metabolome and transcriptome (Kristensen et al. 2005; Kutchan 2005; Memelink 2005; Tattersall et al. 2001). The transgenic *A. thaliana* lines producing high levels (up to 4% dry weight) of the tyrosine-derived dhurrin was generated by two consecutive transformation events. First the two cytochromes P450, CYP79A1 and CYP71E1, were introduced via a single construct, p2x (Fig. 1D; Bak et al. 2000). These transgenic *A. thaliana* lines, designated 2x, appeared stressed and stunted in growth due to accumulation of toxic dhurrin intermediates and derivatives thereof (Fig. 2, panel 2x; Bak et al. 2000; Kristensen et al. 2005; Tattersall et al. 2001). In contrast, *A. thaliana* plants transformed with *S. bicolor CYP79A1* did not display a visual phenotype. Co-transformation of *CYP79A1* with *CYP71E1* generates a new sink for *p*-hydroxyacetaldoxime metabolism and efficiently prevents redirection of the majority of the generated *p*-hydroxyphenylacetaldoxime into the glucosinolate pathway. Accordingly, the *A. thaliana* 2x lines preferentially produce the unstable cyanohydrin *p*-hydroxymandelonitrile which decomposes into primarily *p*-hydroxybenzoic acid via *p*-hydroxybenzaldehyde (Bak et al. 2000; Kristensen et al. 2005; Tattersall et al. 2001). In the 2x lines, the *p*-hydroxybenzoic acid is glucosylated and accumulates as *p*-hydroxybenzoylglucoside (Fig. 2, panel 2x). Unexpectedly, the UV protectants sinapoyl malate and sinapoyl glucose were decreased in the *A. thaliana* 2x lines (Fig. 2; Kristensen et al. 2005).

To complete the dhurrin biosynthetic pathway, *A. thaliana* 2x lines were re-transformed with pGT (Fig. 1D), thereby introducing UGT85B1, the UDP-glucosyl transferase that catalyzes the final step in biosynthesis of dhurrin in *S. bicolor* (Jones et al. 1999). Upon introduction of UGT85B1 (Fig. 1B, C), the visual phenotype was restored to wild type and up to 4% dry weight dhurrin accumulated. As a consequence, levels of sinapoyl malate and sinapoyl glucose were restored to wild type, and the detoxification products observed in the recipient 2x lines were no longer detectable (Fig. 2, panel 3x; Kristensen et al. 2005; Tattersall et al. 2001). Notably, *p*-hydroxybenzylglucosinolate was not detected in the *A. thaliana* 3x lines, indicating that in the presence of the entire set of enzymes catalyzing the dhurrin pathway, the glucosinolate pathway is no longer able to compete for the *p*-hydroxyphenylacetaldoxime generated by *S. bicolor* CYP79A1. A likely explanation is that when *S. bicolor* CYP79A1, CYP71E1, and UGT85B1 are co-expressed, they form a tight metabolon that effectively channels tyrosine to dhurrin (Jørgensen et al. 2005b; Møller and Conn 1980) and simultaneously prevents *S. bicolor* CYP79A1 from interacting with the post oxime enzymes in the glucosinolate pathway. These results prove that it is possible to engineer transgenic plants that produce significant amounts of a novel secondary metabolite and yet adhere to the principle of substantial equivalence (Kristensen et al. 2005).

Despite the high levels of dhurrin, the *A. thaliana* 3x lines are not highly cyanogenic, i.e. they are only able to slowly degrade dhurrin. This reflects the lack of a specific β-glucosidase catalyzing immediate hydrolysis of dhurrin. Consequently, tissue damage results in a cyanide fizz rather than a cyanide bomb (Tattersall et al. 2001). Whereas the cruciferous specialist flea beetle *P. nemorum* did not discriminate between the *A. thaliana* 1x lines that accumulate high levels of *p*-hydroxybenzylglucosinolate and wild type leaves (Nielsen et al. 2001), a significant deterrent effect in choice tests between *A. thaliana* 3x and wild type was observed. The flea beetles consumed up to 80% less of the *A. thaliana* 3x leaf material as compared to wild type (Tattersall et al. 2001). Similarly, the majority of flea beetle larvae died when fed the dhurrin containing 3x lines (Tattersall et al. 2001). Experiments in which the flea beetles were starved for 2 days and subsequently fed dhurrin producing *A. thaliana* 3x plants in non-choice experiments, revealed that the flea beetles did consume leaf material from the cyanogenic plants, but that this resulted in transient paralysis in their legs (our unpublished data). These results unambiguously confirmed that cyanogenic glucosides may confer resistance to herbivores. The results also served to illustrate the inherent ability of animals to detoxify HCN (Zagrobelny et al. 2004) in that paralysis was only transient.

1.3 Expression of the dhurrin biosynthetic pathway in *L. japonicus*

Sorghum bicolor accumulates the aromatic cyanogenic glucoside dhurrin derived from tyrosine while *L. japonicus* accumulates the aliphatic cyanogenic glucosides linamarin and lotaustralin derived from valine and isoleucine, respectively. Expression of *S. bicolor CYP79A1*, *CYP71E1* and *UGT85B1* either separately or in concert in *L. japonicus* would facilitate studies of the flexibility of the cyanogenic glucoside pathway in a cyanogenic plant. This would yield valuable information on the ability of the enzymes of one pathway to interact and enter into a metabolon with enzymes of the parallel pathway, on the substrate specificity of the post oxime enzymes and on the capability of *L. japonicus* to host an entire heterologous pathway and synthesize and store a new cyanogenic glucoside. In addition such plants would facilitate a study of the impact of the total cyanogenic glucoside content as well as the cyanogenic glucoside profile on the interactions between *L. japonicus* and *Zygaena* species. Accordingly the dhurrin pathway was introduced into *L. japonicus*. To achieve this, three approaches were pursued. The first approach was analogous to the introduction of the tyrosine-derived *p*-hydroxybenzylglucosinolate in *A. thaliana* taking advantage of an expected relatively broad substrate specificity of the post oxime enzymes in the endogenous *L. japonicus* cyanogenic glucoside pathway. Previously, microsomes prepared from *L. japonicus* leaves have been shown to convert *p*-hydroxyphenylacetaldoxime into the corresponding cyanohydrin, *p*-hydroxymandelonitrile (Forslund et al. 2004), thus demonstrating that the post oxime enzymes in *L. japonicus* are able to metabolize *p*-hydroxyphenylacetaldoxime. Free oximes are generally known to be toxic to the plant (Bak et al. 1999; Grootwassink et al. 1990; Hemm et al. 2003) and thus the ability of endogenous enzymes to metabolize *p*-hydroxyphenylacetaldoxime is a prerequisite for successful expression of *S. bicolor CYP79A1* in *L. japonicus*. Accordingly, *L. japonicus* were transformed with construct p1x (Fig. 1D) to introduce *S. bicolor* CYP79A1. However, no transformants were obtained that expressed *S. bicolor CYP79A1*. In *L. japonicus*, the cyanogenic glucoside pathway was subsequently shown not be expressed in the callus phase (our unpublished data) as also observed in *M. esculenta* (Joseph et al. 1999). Accordingly, successfully transformed cells or calli would accumulate toxic *p*-hydroxyphenylacetaldoxime and as a consequence most likely die of intoxication or silence the transgene. In retrospect, this approach thus appears suboptimal.

The second approach was based on the ability to transform the three cDNAs encoding the enzymes of the dhurrin pathway into *A. thaliana* (Tattersall et al. 2001). Accordingly, experiments were set up to initially transform *L. japonicus* with the p2x construct (Fig. 1D) with a planned re-transformation with pGT. However, it was not possible to regenerate shoots from the *L. japonicus* 2x explants. The fact that *A. thaliana* but not *L. japonicus* may be successfully transformed with the p2x construct is most probably due to a combination of factors that each imposes a negative selection against p2x transformed *L. japonicus* explants. A major difference relates to the transformation procedure employed. *L. japonicus* transformation requires an extended callus phase (Handberg and Stougaard 1992), in which the plant cells remain in an undifferentiated state, whereas *A. thaliana* is transformed by simply dipping developing flowers into a solution of *Agrobacterium* and subsequently harvesting and selecting the transformed seeds (Clough and Bent 1998). Moreover, *A. thaliana* is able to detoxify a proportion of the tyrosine-derived oxime by redirection into the glucosinolate pathway. Finally, *L. japonicus* 2x explants most likely do not possess the physiological machinery to handle the toxic compounds produced by CYP79A1 and CYP71E1, and are probably subjected to cyanide intoxication. *N. tabacum* and *Vitis vinifera* L. (grapevine), two species that likewise require a callus phase as part of the transformation procedure have been successfully transformed with p2x, and expression of *CYP79A1* and *CYP71E1* obtained (Bak et al. 2000; Franks

et al. 2006). A major difference between these three species is that the callus phase in the course of *N. tabacum* transformation is significantly shorter (~1 month) than the callus phase required for *L. japonicus* transformation (3–6 months), while *V. vinifera* transformation involves an intermediate 2–3 months callus phase (Iocco et al. 2001). In addition, out of 35 *N. tabacum* 2x transformants, only ten lines produced detectable amounts of CYP79A1 and CYP71E1 enzyme activity and with significantly lower enzyme activity compared to *A. thaliana* 2x (Bak et al. 2000). Likewise, only 2 out of 19 kanamycin resistant *V. vinifera* 2x transformants expressed detectable *CYP79A1* and *CYP71E1* (Franks et al. 2006). These results suggest that transformants which expressed high levels and/or highly active CYP79A1 and CYP71E1 are selected against during the callus phase or during regeneration.

In order to circumvent the toxicity of metabolites derived from expression of the two *S. bicolor* cytochromes P450, a third approach was designed in which the two cytochrome P450 encoding cDNAs were inserted subsequent to the *UGT85B1* cDNA encoding the UDP glucosyl transferase. Accordingly, *L. japonicus* was first transformed with pGT, thus introducing *S. bicolor* UGT85B1, to ensure that the toxic cyanohydrin synthesized by the two cytochromes P450 introduced by transformation with p2x (Fig. 1B, D) could be efficiently metabolized into dhurrin. *L. japonicus* lines were readily generated that effectively glucosylated *p*-hydroxymandelonitrile as monitored by an in vitro assay for UGT85B1 activity (Fig. 3A, lane 1). The inability of extracts prepared from wild type *L. japonicus* to glucosylate *p*-hydroxymandelonitrile into dhurrin documented the lack of ability of post oxime enzymes in wild type *L. japonicus* to catalyze the final step in dhurrin biosynthesis. This substantiates the previous observation that it was not possible to achieve dhurrin formation by transformation with p1x or p2x alone (Fig. 1B, D). For re-transformation with p2x introducing the two *S. bicolor* cytochromes P450, an *L. japonicus* GT line that showed high UGT85B1 activity (Fig. 3A, lane 1) and contained one single copy of *S. bicolor UGT85B1* was selected. Transformation of *L. japonicus* is based on explants prepared from hypocotyls (Handberg and Stougaard 1992). As seeds from the primary GT transformant were used for re-transformation, ¾ of the number of seedlings used for re-transformation with p2x were expected to be either heterozygous or homozygous for the *UGT85B1* cDNA. Two *L. japonicus* 3x lines (out of ten) produced dhurrin as monitored by administration of radio labelled tyrosine to detached trefoils (24 h incubation) followed by extraction of the metabolites and analysis by radio TLC (data not shown). Accordingly, the dhurrin pathway can be assembled in *L. japonicus* as also seen in *A. thaliana* and *V. vinifera* (Franks et al. 2006; Tattersall et al. 2001). Unexpectedly, the dhurrin production proved to be transient, and the ability to synthesize dhurrin was lost within 3–4 weeks.

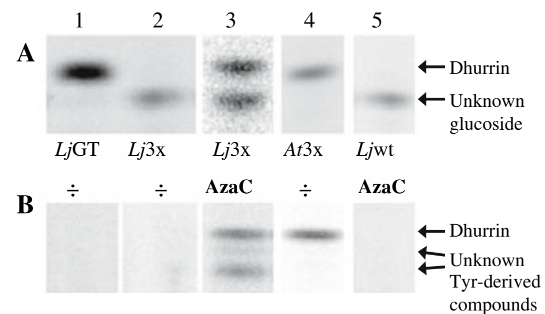


Fig. 3 5′-Azacytidine (azaC) re-activates dhurrin biosynthesis and UGT85B1 activity in silenced *L. japonicus* transformed with the dhurrin pathway from *S. bicolor*. (**A**) UGT85B1 activity in transgenic *L. japonicus* as monitored by administration of ^{14}C-glucose and *p*-hydroxymandelonitrile to leaf extracts. Extracts from *L. japonicus* transformed with *S. bicolor UGT85B1* alone (*Lj*GT) showed high UGT85B1 activity (*lane 1*). When *Lj*GT was re-transformed with *S. bicolor CYP79A1* and *CYP71E1* (*Lj*3x), UGT85B1 activity was not detected in leaf extracts (*lane 2*). In the presence of azaC, UGT85B1 activity in *L. japonicus* 3x extracts was re-activated (*lane 3*). As a positive control, extracts from *A. thaliana* transformed with the entire dhurrin pathway (*At*3x) was included (*lane 4*), and extract from *L. japonicus* wild type (*Lj*wt) was included as a negative control (*lane 5*). An unknown glucoside is synthesized in extracts from all *L. japonicus* lines indicating the presence of an endogenous glucosyl transferase activity. UGT85B1 activity was assayed and analyzed by thin layer chromatography (TLC) as described by Hansen et al. (2003). (**B**) Dhurrin biosynthesis in transgenic *L. japonicus* as monitored by administration of ^{14}C-tyrosine to detached leaves. After 24 h incubation metabolites were extracted with boiling 85% methanol and analyzed by TLC. *L. japonicus* expressing *UGT85B1* alone (*lane 1*) does not synthesize dhurrin from tyrosine (*lane 1*). In the absence of azaC (÷), the silenced *Lj*3x does not accumulate dhurrin (*lane 2*) while in the presence of azaC, *Lj*3x accumulates dhurrin in addition to at least two unknown tyrosine-derived metabolites (*lane 3*). *A. thaliana* 3x (*lane 4*) and *L. japonicus* wild type (*lane 5*) were included as positive and negative controls, respectively. ^{14}C-tyrosine application to monitor dhurrin biosynthesis was performed as described in Bak et al. (2000). For azaC treatment, seeds of *L. japonicus* wild type (negative control) and 3x primary transformants were scarified, sterilized and germinated as described by Forslund et al. (2004) on MS medium (adapted from Murashige and Skoog 1962) supplemented with 50 µg/mL 5′-azacytidine (Sigma, Saint Louis, MO, USA)

1.3.1 *Transgene silencing and re-activation of dhurrin biosynthesis by 5′-azacytidine treatment*

The unexpected elusiveness of dhurrin accumulation in *L. japonicus* 3x plants could result from enzymatic degradation of dhurrin, inhibition of biosynthetic enzyme activity, degradation of the heterologously expressed enzymes, transcriptional gene silencing (TGS), or post TGS (PTGS). In contrast to *A. thaliana*, *L. japonicus* contains endogenous

β-glucosidases which efficiently degrade dhurrin (our unpublished results), and an increased or delayed onset of β-glucosidase activity in the new transgenic plants might cause the sudden apparent lack of dhurrin accumulation. TGS results in decreased transcription and is known to be associated with homology in promoter regions. In contrast, PTGS involves sequence-specific degradation of the transcribed mRNA and correlates with sequence homology in coding regions (Fagard and Vaucheret 2000; Park et al. 1996). The fact that all five transgenes in *L. japonicus* 3x are controlled by identical CaMV 35S promoters (Fig. 1D) while no sequence homology is present in the coding regions strongly implied that TGS was the cause of transient dhurrin production. Methylation of cytidine residues in promoter regions is known to correlate with TGS in plants (Fagard and Vaucheret 2000; Kumpatla and Hall 1998 and references therein). 5′-Azacytidine (azaC) is a cytidine homologue which replaces cytidine in the chromosomal DNA during replication and acts as a potent demethylating agent via inhibition of the DNA methyltransferase (Gabbara and Bhagwat 1995). Accordingly, azaC treatment can be used to relieve methylation-dependent gene silencing. When seeds harvested from the parental transgenic *L. japonicus* lines that transiently produced dhurrin were germinated in the presence of azaC, dhurrin biosynthesis was re-activated as detected by administration of radio labelled tyrosine to detached leaves and TLC analysis (Fig. 3B, lane 3) and by LC-MS (Fig. 4). A negative side effect of treatment with azaC is impaired growth and root development (Fig. 4). The amount of dhurrin accumulated varied with some lines containing dhurrin in amounts approaching the prevalent endogenous cyanogenic glucoside lotaustralin (Fig. 4). Neither dhurrin, nor *S. bicolor* UGT85B1 activity was detected in *L. japonicus* 3x germinated in the absence of azaC or in wild type independent of azaC treatment (Figs. 3, 4). These data demonstrate that the transgene silencing is inherited and caused by DNA methylation which indicates that TGS is the mechanism responsible for transgene silencing.

The ratio of dhurrin producing *L. japonicus* 3x progeny observed when germinated in the presence of azaC corresponds to an expected 9/16 ratio based on simple Mendelian segregation of the two T-DNAs derived from p2x and pGT in a heterozygous parent plant. Re-activation of dhurrin production proved to be transient as silencing recurred in all plants examined at the latest 6 weeks after germination in the presence of azaC. This is most likely linked to the instability of azaC as well as the dilution resulting from repeated cell division. *L. japonicus* is thus able to recognize and inactivate the transgenes once azaC

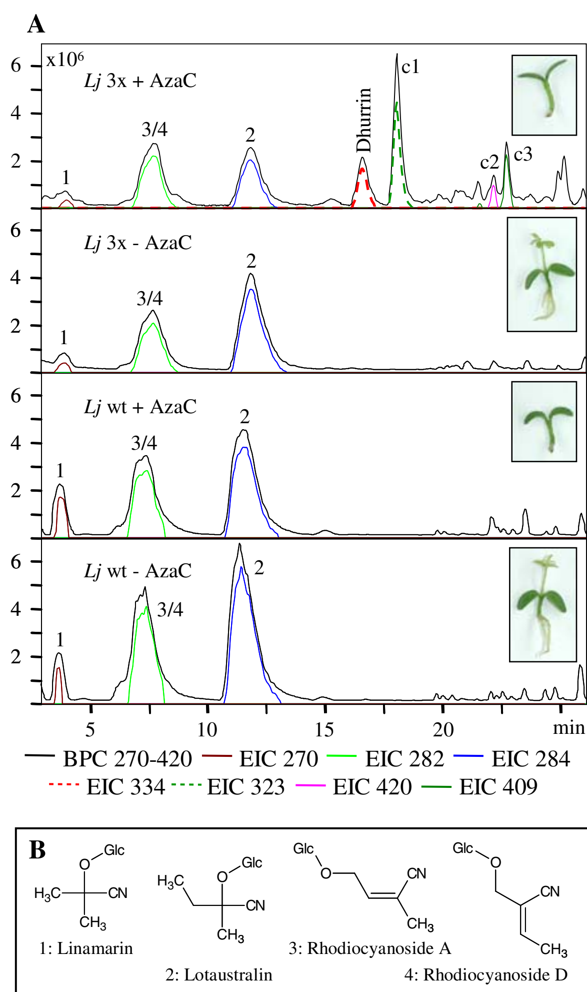


Fig. 4 Metabolic profiles of transgenic (*Lj*3x) and wild type (*Lj*wt) *L. japonicus* germinated in the presence or absence of 5′-azacytidine (azaC). (**A**) LC-MS analysis of *L. japonicus* 3x and wild type. *Lj*3x is transformed with *S. bicolor CYP79A1*, *CYP71E1* and *UGT85B1*. The inserted pictures demonstrate the visual phenotypes of 14-day-old seedlings germinated in the presence (+) or absence (−) of azaC. Base Peak Chromatograms (BPC) 270-420 and selected extracted ion chromatograms (EIC) are shown. AzaC treated *Lj*3x accumulate dhurrin (EIC 334, as confirmed by comparison to an authentic standard and analysis of MS2 spectra) in addition to three unknown tyrosine-derived compounds (c1: EIC 323, c2: EIC 420 and c3: EIC 409). Compounds 1–4 refer to the structures listed in *panel B*. (**B**) Structures of the cyanogenic glucosides linamarin (1: EIC 270) and lotaustralin (2: EIC 284) and the cyanoalkenyl glucosides, rhodiocyanoside A (3: EIC 282) and rhodiocyanoside D (4: EIC 282). Metabolites were extracted in 85% (v/v) boiling methanol for 2 min, filtered and subjected to LC-MS analysis. Analytical LC-MS was carried out using an Agilent 1100 Series LC (Agilent Technologies, Germany) coupled to a Bruker Esquire 3000+ ion trap mass spectrometer (Bruker Daltonics, Bremen, Germany). An XTerra MS C18 column (Waters, Milford, MA, USA; 3.5 µM, 2.1 mm × 100 mm) was used at a flow rate of 0.2 mL min^{-1}. The mobile phases were: (1) water with formic acid (0.1% v/v) and sodium chloride (50 µM); (2) acetonitrile/water (80% v/v MeCN) with formic acid (0.1%). The gradient program was: 0–4 min, isocratic 2% B; 4–10 min, linear gradient 2–8% B; 10–30 min, linear gradient 8–50% B; 30–35 min, linear gradient 50–100% B; 35–40 min, isocratic 100% B. The mass spectrometer was run in electrospray mode and positive ions were observed

levels have been diluted below a certain threshold. Re-activation of silenced transgenes in rice by azaC treatment followed by new onset of silencing was similarly observed in the study by Kumpatla and Hall (1998).

Transcriptional gene silencing, however, does not exclude PTGS as a determining factor in silencing of the dhurrin pathway in *L. japonicus* 3x. To examine a possible additional effect of PTGS, *L. japonicus* 3x could be transformed with a viral suppressor of PTGS such as the p19 protein from tomato bushy stunt virus. The p19 protein is well known to enhance expression of transgenes in *N. tabacum* (Voinnet et al. 2003). Studying the effect of p19 in the presence and absence of azaC might reveal any additive effect of PTGS in the observed silencing. We have not pursued this possibility as it would necessitate a transformation round and a subsequent laborious screening for lines that successfully express all three cDNA constructs in a population segregating for three traits.

In addition to dhurrin, two new tyrosine-derived compounds were detected upon administration of radio-labelled tyrosine to detached leaves of *L. japonicus* 3x germinated in the presence of azaC (Fig. 3B, lane 3). In comparison, dhurrin was the only new metabolite detected in *A. thaliana* 3x leaves [Figs. 2 (panel 3x), 3B (lane 4)]. LC-MS analysis revealed that azaC treated *L. japonicus* 3x produced three new unknown compounds in addition to dhurrin (m/z 311) [Fig. 4, (c1, c2 and c3)] with m/z of 300, 397 and 386, respectively. The UV spectra recorded indicated that c1, c2 and c3 are aromatic and derived from tyrosine (results not shown) in agreement with the results obtained when radio-labelled tyrosine was administered (Fig. 3B). As none of the three unknown compounds were detected in extracts from *L. japonicus* 3x germinated in the absence of azaC, nor in wild type *L. japonicus* germinated with or without azaC (Fig. 4), this strongly indicates that c1, c2 and c3 are related to the inserted dhurrin pathway.

1.3.2 *Why does L. japonicus silence the dhurrin pathway?*

Transgene silencing was not observed in *L. japonicus* 35S::*CYP79D2* (Forslund et al. 2004) nor in *L. japonicus* 35S::*UGT85B1* primary transformants. These *L. japonicus* lines each held two copies of the CaMV 35S promoter. The most likely cause of the silencing observed in *L. japonicus* 3x is homology-dependent TGS due to the presence of at least five copies of the CaMV 35S promoter that drive the expression of the transgenes and the selective marker genes (Fig. 1D). This explanation is supported by the fact that *S. bicolor* UGT85B1 is highly active in this *L. japonicus* GT line but becomes silenced upon re-transformation with *S. bicolor CYP79A1* and *CYP71E1* (Fig. 3A, lanes 1, 2). In agreement with this hypothesis, strong *S. bicolor* UGT85B1 activity was observed in false positive transformants (*L. japonicus* GT lines that escaped kanamycin selection) which indicates that loss of GT activity was caused by introduction of *S. bicolor CYP79A1* and *CYP71E1* and not by passing through a second callus phase. In the attempt to introduce dhurrin into *V. vinifera* hairy roots, accumulation of *S. bicolor CYP79A1* and *CYP71E1* transcripts was detected in *V. vinifera* 2x (Franks et al. 2006). Upon re-transformation with *S. bicolor UGT85B1*, some transgenic lines produced dhurrin while in others, all three transgenes were silenced. Hence re-transformation with *S. bicolor UGT85B1* silenced the previously expressed *S. bicolor CYP79A1* and *CYP71E1*. In analogy with our results, the authors suggested that the lack of transgene expression was a result of homology-dependent TGS caused by the presence of multiple CaMV 35S promoters (Franks et al. 2006).

In conclusion, the main differences between *A. thaliana* 3x and *L. japonicus* 3x transgenic lines are that *A. thaliana* 3x was transformed by the floral dip method (Clough and Bent 1998) and initially with p2x followed by pGT, while *L. japonicus* transformation involved an extended callus phase (Handberg and Stougaard 1992) and initial transformation with pGT followed by re-transformation with p2x. The *A. thaliana* 3x transgenic lines studied by Kristensen et al. (2005) were homozygous for the transgenes and hence held ten copies of the CaMV 35S promoter. As transformation of *A. thaliana* with *CYP79A1* and *CYP71E1* results in a severe phenotype, the 2x transformants are likely to have been selected for low amounts or reduced activity of the two cytochromes P450. In agreement with this, DNA microarray data showed that *CYP79A1*, *CYP71E1* as well as *UGT85B1* were expressed at low levels in spite of being expressed under control of the CaMV 35S promoter (Kristensen et al. 2005). These data indicate that even low concentrations of the transcripts encoding the dhurrin biosynthetic enzymes are sufficient for production of high amounts of dhurrin. In contrast, the *L. japonicus* GT line chosen for re-transformation with p2x had high GT activity (Fig. 3A, lane 1). These results indicate that low enzyme activity might be preferable for production of dhurrin in a heterologous plant species and that choice of a low UGT85B1 activity *L. japonicus* line for p2x transformation line might have been preferable in order to avoid transgene silencing.

1.4 Changing the cyanogenic glucoside profile in transgenic *M. esculenta* and *L. japonicus*

1.4.1 *The quest for acyanogenic M. esculenta*

In order to produce *M. esculenta* with acyanogenic tubers ultimately aimed for safe consumption, two approaches were pursued to reduce the content of cyanogenic

glucosides (Jørgensen et al. 2005a). An enhanced CaMV 35S controlled antisense construct was designed to target the first committed step in the pathway. As in other cyanogenic plants, this step is catalyzed by a cytochrome P450 belonging to the CYP79 family (Andersen et al. 2000), but as *M. esculenta* is allopolyploid it holds two *CYP79* copies, *CYP79D1* and *CYP79D2*, which are co-expressed as determined by *in tube* in situ PCR (Jørgensen et al. 2005a). Both *CYP79D1* and *CYP79D2* need to be targeted in a single construct as they both catalyze the first committed step in cyanogenic glucoside biosynthesis in *M. esculenta* (Andersen et al. 2000). Transgenic *M. esculenta* lines with up to 80% reduction in cyanide potential in the first unfolded leaf and a 60% reduction of cyanogenic glucoside content in tubers were obtained using antisense technology (Jørgensen et al. 2005a). To obtain an even higher reduction in cyanogenic glucoside content, an RNAi hairpin construct targeting *CYP79D1* and *CYP79D2* was designed. The RNAi construct under control of the enhanced CaMV 35S promoter was transformed into *M. esculenta* and resulted in >99% reduction in leaf cyanogenic glucoside content in plants grown in vivo (Jørgensen et al. 2005a). Figure 5 shows the content of linamarin in leaves of a representative selection of antisense and RNAi *M. esculenta* transformants as compared to wild type. Silencing cyanogenic glucoside biosynthesis by antisense technology results in *M. esculenta* transformants with leaf linamarin contents ranging between 20 and 130% of wild type levels (Fig. 5A), whereas near acyanogenic *M. esculenta* lines were obtained using RNAi technology, with leaf linamarin content ranging from less than 1 to 250% of wild type levels (Fig. 5B). The mechanism that results in the apparent up regulation of cyanogenic glucoside accumulation in some lines upon transformation with antisense or RNAi constructs remains unclear.

The low cyanogen (<25% of wild type cyanide potential) *M. esculenta* RNAi transformants had long, slender stems with long internodes when grown in vitro. When the low cyanogen lines where moved to high nitrogen media or to soil, the phenotype was complemented (Jørgensen et al. 2005a, b). The restored visual phenotype was not due to increased accumulation of cyanogenic glucosides, given that ~90% of the soil grown RNAi *M. esculenta* lines retained the >99% reduction in leaf cyanogenic glucoside content as compared to wild type. Apart from a direct involvement in plant defense, cyanogenic glucosides have also been claimed to act as nitrogen storage compounds (Selmar et al. 1988). The phenotypes observed when grown in low nitrogen media might be a reflection of perturbed nitrogen homeostasis or even that cyanogenic glucosides act as signalling compounds that can affect enzyme activities or gene expression involved in plant development. Alternatively, the RNAi construct might have targeted other transcripts in addition to *CYP79D1* and *CYP79D2* affecting in vitro growth. Future experiments using *M. esculenta* oligonucleotide DNA micro arrays might help to elucidate the impact of linamarin and lotaustralin on *M. esculenta* fitness and development.

In the *M. esculenta* RNAi lines, the cyanogenic glucoside content in tubers varied from 8% to more than 200% of wild type levels in spite of the <1% cyanogenic glucoside content observed in the leaves (Jørgensen et al. 2005a). In *M. esculenta*, the cyanogenic glucosides are primarily synthesized in leaves and transported to the tubers (Jørgensen et al. 2005a). The accumulation of high amounts of cyanogenic glucosides in tubers from plants with almost acyanogenic leaves indicates a very efficient transport of cyanogenic glucosides from leaves to tubers, and supports that *de novo* synthesis also takes place in roots

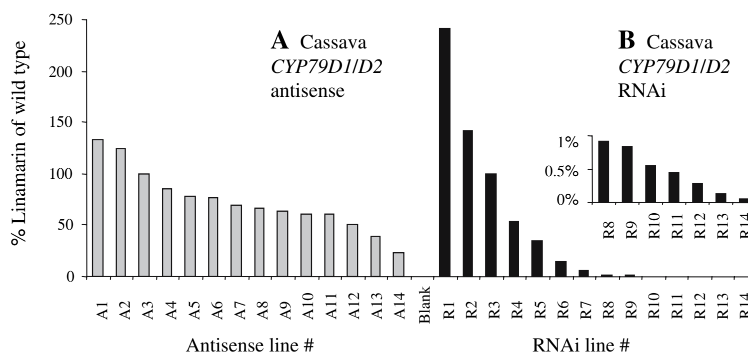


Fig. 5 *Manihot esculenta* lines with altered cyanide profile generated by antisense (A) and RNAi (B) technology. Antisense and RNAi constructs were targeted against *CYP79D1* and *CYP79D2* that encode the enzymes catalyzing the first committed steps in cyanogenic glucoside biosynthesis in *M. esculenta* (Andersen et al. 2000). Linamarin contents in MeOH extracts prepared from the first unfolded leaves were quantified by LC-MS. A representative selection of transgenic *M. esculenta* lines are shown to illustrate the distribution of leaf linamarin content in antisense (A) and RNAi (B) lines compared to wild type. Anti-sense and RNAi constructs targeted against *CYP79D1* and *CYP79D2*, and *M. esculenta* plants with depleted linamarin and lotaustralin contents were produced and analyzed as described by Jørgensen et al. (2005a)

of *M. esculenta* (Du et al. 1995). In addition, a reduced rate of catabolism of cyanogenic glucosides could contribute to the accumulation of cyanogenic glucosides in the tubers of otherwise acyanogenic *M. esculenta*.

The CaMV 35S promoter is generally regarded as a constitutive, highly active promoter that is active in most plant cell types though numerous plant species dependent exceptions have been reported (e.g. Samac et al. 2004). In *M. esculenta*, the CaMV35S promoter is known to have reduced activity in the root cells where the *CYP71E1* orthologue is expressed (Zhang et al. 2003). Accordingly, a promoter that specifically drives expression at the cellular sites of cyanogenic glucoside biosynthesis is desirable to obtain *M. esculenta* with acyanogenic tubers. Work is in progress to engineer a *CYP79D1*/*CYP79D2* RNAi construct under control of an endogenous promoter that specifically controls expression of cyanogenic glucoside biosynthesis to facilitate a more targeted silencing. With respect to the use of *M. esculenta* as a source of food for humans, an optimal transgenic *M. esculenta* plant would most likely be a plant which produces acyanogenic tubers for consumption while retaining at least a medium cyanide potential in the aerial parts to protect against pests. Engineering of such a *M. esculenta* variety would require additional knowledge on the genes controlling transport of cyanogenic glucosides, their biosynthesis in roots and possibly the activity of cyanogenic glucoside degrading β-glucosidases.

1.4.2 *CYP79D3 and CYP79D4 as targets in the production of L. japonicus with reduced cyanogenic glucoside and rhodiocyanoside content*

Lotus japonicus is diploid, and yet it holds two *CYP79D* paralogues, *CYP79D3* and *CYP79D4*, which encode the enzymes that catalyze the first committed steps in cyanogenic glucoside and rhodiocyanoside biosynthesis in *L. japonicus* (Forslund et al. 2004). The presence of two *CYP79D* paralogues in *L. japonicus* is probably a result of a gene duplication followed by sub-functionalization resulting in a differential expression of the two genes (Forslund et al. 2004). *CYP79D3* is expressed in aerial tissue and *CYP79D4* in roots as determined by RT-PCR (Forslund et al. 2004). To enable a study of the impact of cyanogenic glucosides and rhodiocyanosides in *L. japonicus*, an RNAi construct targeted against both *CYP79D3* and *CYP79D4* under control of an enhanced CaMV 35S promoter was transformed into *L. japonicus*. Out of 11 independent transgenic *L. japonicus* RNAi lines, only two showed a reduction in cyanogenic glucoside and rhodiocyanoside content in leaves as compared to wild type (Fig. 6). These results strongly support that CYP79D3 and/or CYP79D4 provide precursors for the synthesis of rhodiocyanosides in addition to cyanogenic glucosides in *L. japonicus* (Forslund et al. 2004). LC-MS analysis of metabolite content was initially performed on the first leaves that appeared on the transformed plants after they had been transferred from in vitro culture to hydroponics in the green house (Fig. 6, upper panel). When the analysis was repeated with new leaves from the same plants 8 weeks later, lotaustralin and rhodiocyanoside contents had increased to wild type levels (Fig. 6, lower panel). The apparent inability to reduce cyanogenic glucoside and rhodiocyanosides levels might reflect the rate of catabolism is regulated to approach wild type levels. Cyanogenic glucosides and rhodiocyanosides may also play an important developmental or physiological role in *L. japonicus* and if this causes a negative correlation between the ability to regenerate plants and their selection during transformation, the chance of producing efficiently silenced lines by RNAi technology would be very low.

As an alternative to RNAi technology, isolation of Targeted Induced Local Lesions IN Genomes (TILLING) mutants of *L. japonicus* is now a possibility with the generation of an *L. japonicus* TILLING collection (http://www.lotusjaponicus.org/tillingpages/homepage.htm). Identification of *L. japonicus* lines with mutations in *CYP79D3* and *CYP79D4* or in the as yet unknown transcription factor that regulates their expression might yield truly acyanogenic *L. japonicus* lines. This would provide useful tools to dissect unknown functions of cyanogenic glucosides and rhodiocyanosides in plant fitness and adaption to biotic and abiotic stresses. They may also approve valuable in unravelling the impact of cyanogenic glucosides and rhodiocyanosides in interactions with *Zygaena* larvae and plant development (Zagrobelny et al. 2004; Zagrobelny et al. 2007).

1.5 Expression of *M. esculenta CYP79D2* in *L. japonicus* results in increased accumulation of linamarin but not lotaustralin

Lotus japonicus accumulates low levels of valine-derived linamarin and high levels of isoleucine-derived lotaustralin and rhodiocyanoside A and D (Forslund et al. 2004). This is in contrast to other *Lotus* species such as *L. corniculatus* which accumulate an approximate 1:1 ratio of linamarin to lotaustralin (Zagrobelny et al. 2004; Zagrobelny et al. 2007 and our unpublished results). To generate *L. japonicus* with an increased level of linamarin, *M. esculenta CYP79D2* was expressed under control of the CaMV 35S promoter. Using this strategy, production of *L. japonicus* lines with an up to $\sim$20-fold increase in linamarin levels in leaves was achieved while the levels of lotaustralin and rhodiocyanoside A and D were maintained (Fig. 7;

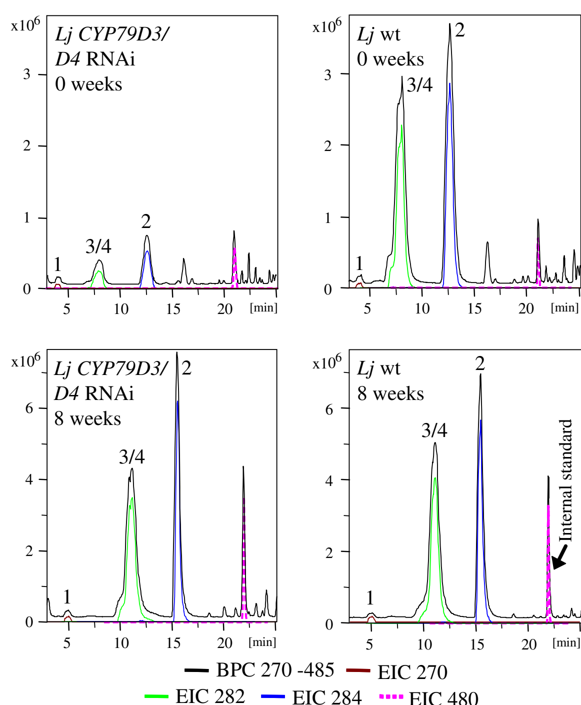


Fig. 6 Transient reduction of lotaustralin and rhodiocyanoside content mediated by RNAi in *L. japonicus*. The RNAi construct employed targets *CYP79D3* and *CYP79D4* which encode the enzymes that catalyze the first committed step in cyanogenic glucoside- and rhodiocyanoside biosynthesis in *L. japonicus* (Forslund et al. 2004). MeOH extracts of the first unfolded leaves were analyzed by LC-MS as detailed in the legend for Fig. 4. Base Peak Chromatograms (BPC) 270-485 and Extracted Ion Chromatograms (EIC) for linamarin (1: EIC 270), rhodiocyanoside A and D (3/4: EIC 282), lotaustralin (4: EIC 284) and amygdalin (internal standard: EIC 480) are shown. *Numbers 1–4* refer to the compounds shown in Fig. 4B. At the time of appearance of the first leaves of the new transgenic plants (*Lj CYP79D3/D4* RNAi, 0 weeks), lotaustralin and rhodiocyanosides are reduced to around ~20% of the level found in wild type (wt). After 8 weeks, the amount of lotaustralin and rhodiocyanosides in the same plants appeared comparable to wild type levels. To generate the RNAi lines, a hairpin loop construct targeted against *L. japonicus CYP79D3* and *CYP79D4* was generated using inverted repeats of a 241 bp DNA fragment with 100% sequence identity between *CYP79D3* and *CYP79D4* (position 386–626 in *CYP79D3*). The inverted repeats were separated by a 349 bp truncated version of the *CYP79D3* intron (positions 943–1,121 and 2,822–2,991 separated by an introduced *Eco*RI restriction site) and ligated between the enhanced CaMV 35S promoter and terminator in pPS48 using the *Bam*HI and *Xma*I restriction sites. The E35S:RNAi:35St construct was subsequently ligated into the *Xba*I site of pCAMBIA 2301 to yield pD3D4RNAi

Forslund et al. 2004). As CYP79D3 and CYP79D4 both catalyze the conversion of valine as well as isoleucine to the corresponding oximes, the specific accumulation of linamarin upon expression of *CYP79D2* indicates an inherent control of lotaustralin and rhodiocyanosides steady state levels in *L. japonicus. L. japonicus* CYP79D3 and CYP79D4 have a ~sixfold higher catalytic efficiency towards isoleucine as compared to valine, which results in the low linamarin to lotaustralin ratio in leaves (Forslund

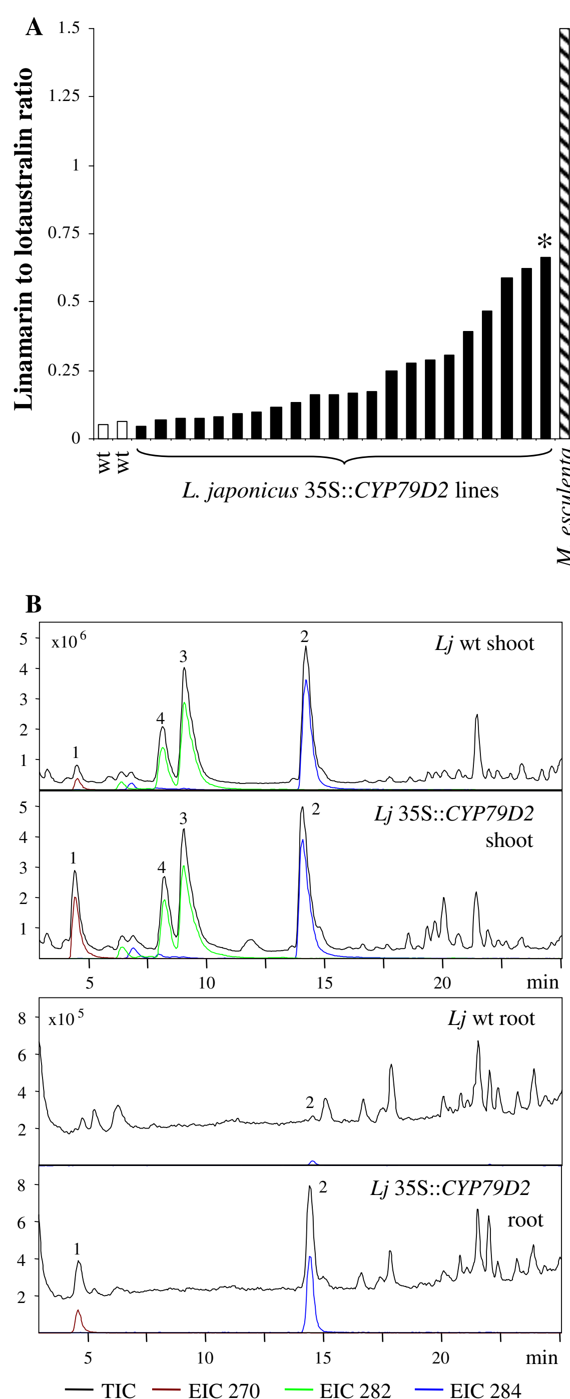


Fig. 7 Expression of *M. esculenta CYP79D2* alters the linamarin to lotaustralin ratio in *L. japonicus*. (**A**) Ratio of linamarin to lotaustralin in *L. japonicus* lines expressing *M. esculenta CYP79D2* as compared to the ratio observed in *M. esculenta*. (**B**) LC-MS analyses of MeOH extracts from leaves and roots of *L. japonicus* wild type (*Lj*wt) and the *L. japonicus* 35S::*CYP79D2* line marked with an *asterisk* in (**A**). Expression of *M. esculenta CYP79D2* in *L. japonicus* results in an up to ~20-fold increase in linamarin (1: EIC 270) accumulation while rhodiocyanoside (3/4: EIC 282) and lotaustralin (2: EIC 284) contents are unaltered in leaves. In roots, linamarin and lotaustralin but not rhodiocyanosides accumulate in the transgenic lines. *Numbers 1–4* refer to the compounds shown in Fig. 4B. The methods applied are detailed in Forslund et al. (2004)

et al. 2004). In contrast to *L. japonicus* which is a diploid, *M. esculenta* is allopolyploid and as a result holds two *CYP79D* copies, *CYP79D1* and *CYP79D2*. The *M. esculenta* CYP79Ds, as determined for CYP79D1, convert isoleucine at ~60% of the conversion rate for valine (Andersen et al. 2000), and thus generate an approximate 3:2 ratio of linamarin to lotaustralin in this species (Forslund et al. 2004). The transgenic *L. japonicus* lines accumulated ~20-fold more linamarin and approached the linamarin to lotaustralin ratio observed in *M. esculenta* (Fig. 7A). This demonstrates that the CYP79 catalyzed enzymatic step exerts quantitative and qualitative control over the flux through the cyanogenic glucoside pathway.

A surprising result of the expression of *M. esculenta CYP79D2* in *L. japonicus* was therefore the accumulation of linamarin and lotaustralin but not rhodiocyanosides in roots (Fig. 7B). This demonstrates that roots could have an inherent capacity to synthesize cyanogenic glucosides but not rhodiocyanosides which strongly indicates the presence of two separate biosynthetic pathways for cyanogenic glucosides and rhodiocyanosides in roots and aerial tissues in *L. japonicus* in accordance with the differential expression of *CYP79D3* and *CYP79D4* (Forslund et al. 2004). The possibility that the root content of linamarin and lotaustralin was derived from translocation from the aerial parts to the roots cannot be ruled out. Unfortunately, the *CYP71E1* orthologues encoding the putative cytochromes P450 that catalyzes the conversion of oxime into cyanohydrin in *L. japonicus* have not yet been identified and this hampers the understanding of how *L. japonicus* organizes the biosynthesis of cyanogenic- and cyanoalkenyl glucosides in aerial tissues; either as parallel metabolons or as a single promiscuous metabolon that synthesizes both sets of glucosides simultaneously.

When *Z. filipendulae* larvae were reared on *L. japonicus* wild type and *L. japonicus* 35S::*CYP79D2*, an increase in linamarin to lotaustralin ratio was observed in the larvae fed on *L. japonicus* 35S::*CYP79D2* compared to wild type (Zagrobelny et al. 2007). These results demonstrate that the linamarin to lotaustralin ratio present in *Zygaena* larvae partly reflects the ratio in their dietary plants (Zagrobelny et al. 2007).

2 Concluding remarks

Predictive metabolic engineering of secondary metabolites is the key goal of many research programs. Yet in most cases, metabolic engineering of secondary metabolites is still in its infancy and metabolic engineering is subject to much trial and error. The lessons learned from metabolic engineering of cyanogenic glucosides may provide clues on how to proceed in other similar research initiatives and highlight important factors to be considered when plants are engineered with the purpose of obtaining altered profiles of their secondary metabolites.

The choice and number of different promoters that control expression of the transgenes appears to be critical, depending on the species. In some plant species several copies of the same promoter may be introduced without any adverse effects, while in other species this may be detrimental to the desired outcome of the experiment. This is exemplified by homozygous *A. thaliana* 3x plants that maintain transgene expression in the presence of ten copies of the CaMV 35S promoter and accumulate high levels of dhurrin (Tattersall et al. 2001). In contrast, heterozygous transgenic *L. japonicus* 3x plants harboring the same constructs but only half the copy number (this paper) are subject to gene silencing. At the other end of the scale is the consistent silencing of transgenes driven by the CaMV 35S promoter in gentian (*Gentiana triflora* × *G. scabra*) (Mishiba et al. 2005).

In the case of *L. japonicus*, application of the *CYP79D3* promoter regulating endogenous cyanogenic glucoside biosynthesis (Forslund et al. 2004) would be an obvious alternative to CaMV 35S for driving expression of the three *S. bicolor* cDNAs that encode the enzymes involved in dhurrin biosynthesis. The risk of TGS due to the presence of several copies of the same promoter could be significantly reduced by expression of a multigene expression construct consisting of the genes encoding the three biosynthetic enzymes and the selective marker under control of one single promoter. By separation of the four proteins with the viral 2A peptide (El Amrani et al. 2004), the individual enzymes are predicted to be co-translationally cleaved to yield CYP79A1, CYP71E1, UGT85B1 and the selective marker protein. Alternatively, or in combination with the 2A polyprotein strategy, the use of an inducible promoter would allow selection of transgenic explants without the risk of counter selection for reduced expression of the transgenes. The use of a wound inducible promoter would add a new dimension to the study of cyanogenic glucosides and their impact on plant–insect interactions by changing their characteristics from being regarded as phytoanticipins (preformed defense compounds) to phytoalexins (defense compounds synthesized in response to herbivore or pathogen attack).

Sorghum bicolor CYP79A1 and *CYP71E1* were transformed into *A. thaliana*, *N. tabacum* and *L. japonicus* with the anticipation that an endogenous UDP glucosyl transferase possessing broad substrate specificity (Hansen et al. 2003; Jones et al. 1999) would readily glucosylate the product *p*-hydroxymandelonitrile to yield dhurrin. This was not the case for any of these plants, which emphasizes the specificity of *S. bicolor* UGT85B1 and the need for insertion of the entire dhurrin biosynthetic pathway in the

transformation experiments. The highly specific *S. bicolor* UGT85B1 glucosyl transferase fulfills the requirement for high substrate specificity combined with the ability to form a metabolon with the two cytochromes P450 in the pathway (Jones et al. 2000; Jørgensen et al. 2005b; Møller and Conn, 1980).

Cyanogenesis is the ability of a living organism to release toxic HCN. Cyanogenesis requires the presence, i.e. the ability of the living organism to synthesize the cyanogenic compound as well as the presence of enzymes that are able to cleave this compound with a concomitant release of HCN (Conn, 1980). In a previously non-cyanogenic plant, introduction of cyanogenesis would therefore typically require not only the introduction of the enzymes involved in biosynthesis but also the catabolic enzymes to facilitate a rapid HCN release. In addition, the cyanogenic compounds produced should be compartmentalized separately from the degrading enzymes until tissue disruption. Though β-glucosidases are present in *A. thaliana* plants, the lack of a specific dhurrin β-glucosidase in *A. thaliana* 3x compromises the utility of these transgenic plants for the study of plant–insect interactions because the cyanide release resulting from tissue damage is slow in comparison to that observed in naturally occurring cyanogenic plants. In order to fully exploit the HCN potential of the *A. thaliana* 3x lines, a cDNA encoding a β-glucosidase with high activity towards dhurrin is currently being introduced into *A. thaliana* 3x.

The major lesson learned from metabolic engineering of cyanogenic glucosides is that detailed knowledge of biosynthesis, regulation, transport, degradation and metabolic cross-talk is a prerequisite for performing predictive metabolic engineering. Even possessing this information, the facility of changing the metabolome of a given plant also depends on the choice and number of promoters in concert with the plant's ability to successfully produce active heterologous enzymes and accommodate the biosynthetic product and possible toxic intermediates thereof.

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