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A combined biochemical screen and TILLING approach identifies mutations in *Sorghum bicolor* L. Moench resulting in acyanogenic forage production

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Summary

Cyanogenic glucosides are present in several crop plants and can pose a significant problem for human and animal consumption, because of their ability to release toxic hydrogen cyanide. *Sorghum bicolor* L. contains the cyanogenic glucoside dhurrin. A qualitative biochemical screen of the M2 population derived from EMS treatment of sorghum seeds, followed by the reverse genetic technique of Targeted Induced Local Lesions in Genomes (TILLING), was employed to identify mutants with altered hydrogen cyanide potential (HCNp). Characterization of these plants identified mutations affecting the function or expression of dhurrin biosynthesis enzymes, and the ability of plants to catabolise dhurrin. The main focus in this study is on acyanogenic or low cyanide releasing lines that contain mutations in CYP79A1, the cytochrome P450 enzyme catalysing the first committed step in dhurrin synthesis. Molecular modelling supports the measured effects on CYP79A1 activity in the mutant lines. Plants harbouring a P414L mutation in CYP79A1 are acyanogenic when homozygous for this mutation and are phenotypically normal, except for slightly slower growth at early seedling stage. Detailed biochemical analyses demonstrate that the enzyme is present in wild-type amounts but is catalytically inactive. Additional mutants capable of producing dhurrin at normal levels in young seedlings but with negligible leaf dhurrin levels in mature plants were also identified. No mutations were detected in the coding sequence of dhurrin biosynthetic genes in this second group of mutants, which are as tall or taller, and leafier than nonmutated lines. These sorghum mutants with reduced or negligible dhurrin content may be ideally suited for forage production.

Introduction

Tolerance to drought, elevated temperatures and high water-use efficiency, make sorghum an ideal cereal crop for arid and semi-arid regions of the world. Over 40 million ha of grain sorghum was harvested globally in 2009 (http://faostat.fao.org/site/567/default.aspx#ancor, accessed 2 December 2010). Forage sorghum is grown widely as a drought-resistant alternative to maize in more arid subtropical to warm temperate regions and has potential as a source for biofuel production on marginal lands not appropriate for food production. Like maize, sorghum is a C4 plant with the attributes of improved photosynthetic efficiency and reduced water loss in hot and dry environments, important traits in the context of current global climate change, limited availability of fertilizer and reduced availability of arable land (Ghannoum, 2009; Gregory *et al.*, 2005).

Several plants, including sorghum produces cyanogenic glucosides (Jones, 1998), which act as defence compounds towards herbivores and pests (Tattersall *et al.*, 2001; Gleadow and Woodrow, 2002a; Møller, 2010a) but may also function as an important antioxidant and play a role in nitrogen turnover and storage (Gleadow and Woodrow, 2000, 2002a,b; Jørgensen *et al.*, 2005a; Kongsawadworakul *et al.*, 2009; Møller, 2010b). In sorghum, the cyanogenic glucoside, dhurrin is derived from tyrosine and is found at higher concentrations in seedlings and young developing leaves compared with mature tissue (Akazawa *et al.*, 1960; Halkier and Møller, 1989; Wheeler *et al.*, 1990; Busk and Møller, 2002). Upon browsing by animals or insects, dhurrin can be hydrolysed by β-glucosidases present in the disrupted leaf tissue, resulting in the release of hydrogen cyanide (HCN), a process termed cyanogenesis (Morant *et al.*, 2008). In response to environmental stresses, which restrict plant growth, such as drought (Wheeler and Mulcahy, 1989), or following nitrogen application (Busk and Møller, 2002) dhurrin concentration typically increases, posing a major threat to livestock. In Australia alone, farmers' reticence to use stressed sorghum as animal fodder decreases the value of the crop by approximately $20 million p.a. (P. Stuart, unpubl. data). Therefore, eliminating the toxicity issues through the development of sorghum varieties with highly reduced or no capacity to produce dhurrin is a key agronomical challenge in sorghum breeding.

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Naturally occurring acyanogenic individuals are found in a number of cyanogenic plant species, for example white clover (*Trifolium repens*) (Olsen *et al.*, 2007, 2008), eucalypts (Gleadow *et al.*, 2003) and bird's-foot trefoil (*Lotus corniculatus*; Zagrobelny *et al.*, 2007), demonstrating that cyanogenic glucosides do not play an essential role in primary metabolism but that their involvement in N turnover and storage serves to fine tune primary metabolism under certain circumstances (Kongsawadworakul *et al.*, 2009; Jørgensen *et al.*, 2005a; Møller, 2010b). In white clover, the selective pressures of protection from herbivory in cyanogenic individuals and increased fitness under cold temperatures in acyanogenic individuals are thought to maintain the polymorphism in the *Ac* (CYP79A1 homologue) and *Li* (β-glucosidase) loci, which determines the functional cyanogenic pathway (Olsen *et al.*, 2008). Acyanogenic individuals have not been identified among wild or cultivated populations of sorghum and to date efforts to breed acyanogenic sorghum lines using natural genetic variation have not been successful (Haskins and Gorz, 1986; Duncan, 1996).

Cyanogenesis in sorghum has been studied extensively at the molecular and biochemical levels (Sibbesen *et al.*, 1994; Koch *et al.*, 1995; Kahn *et al.*, 1997; Bak *et al.*, 1998; Jones *et al.*, 1999). Dhurrin synthesis (Figure 1) involves two cytochrome P450s (CYP79A1 and CYP71E1) and one UDP-glucosyltransferase (UGT85B1) (Møller and Conn, 1979; Jones *et al.*, 1999; Bak *et al.*, 2006). These enzymes function co-operatively within a metabolon which allows labile and toxic intermediates to be channelled into dhurrin formation and prevents undesired metabolic cross-talk (Jørgensen *et al.*, 2005b; Kristensen *et al.*, 2005; Nielsen *et al.*, 2008). The identification of all three biosynthetic genes in sorghum has also facilitated attempts to identify the corresponding genes in other species such as cassava (*Manihot esculenta* Cranz) and the model legume *Lotus japonicus* (Forslund *et al.*, 2004; Jørgensen *et al.*, 2005a, 2011). In the present study, knowledge of the gene sequences required for dhurrin synthesis in sorghum was exploited to undertake a TILLING program to generate and identify mutations in the dhurrin biosynthesis enzymes.

TILLING has been used to identify mutations in particular genes of interest in several crop species such as maize, rice and barley (Till *et al.*, 2004, 2007; Talame *et al.*, 2008; Lababidi *et al.*, 2009). Recently, Xin *et al.* (2008) documented the feasibility of TILLING in sorghum by screening a mutagenized population of 768 sorghum plants for alterations in

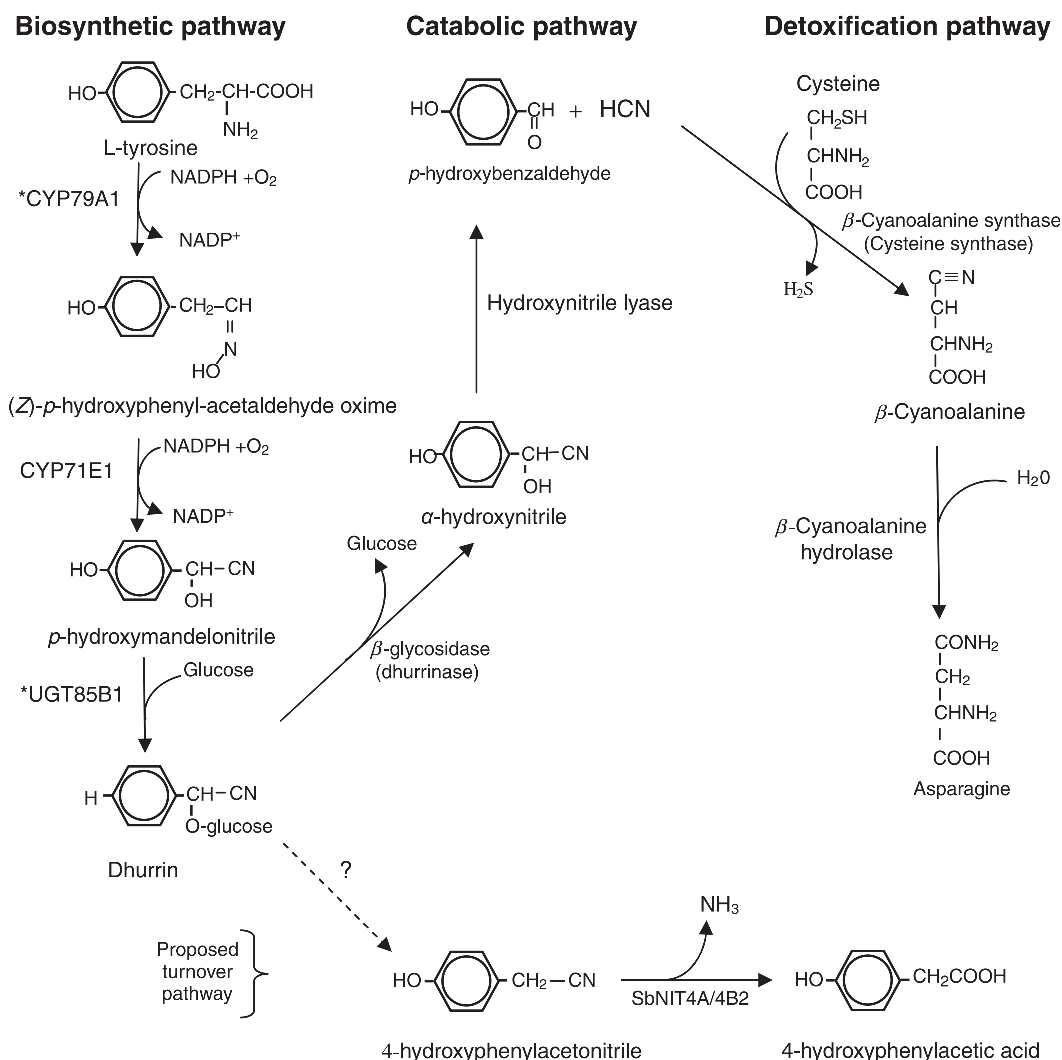

Figure 1 The biosynthetic and detoxification pathways of dhurrin in forage sorghum (from Busk and Møller, 2002; Kristensen *et al.*, 2005; Jenrich *et al.*, 2007).

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genes of agronomic value not associated with cyanogenesis. Cyanogenesis-deficient (*cyd*) mutants were obtained in *L. japonicus* using TILLING with mutations in the β-glucosidase gene BGD2 identified and characterized (Takos *et al.*, 2010). In the present study, targeting genes for disrupted β-glucosidase enzyme function was not considered suitable for the production of an acyanogenic forage sorghum crop as cattle contain micro-organisms in their rumen possessing β-glucosidase activity that would release HCN from dhurrin ingested by the animal regardless of the absence of *in planta* β-glucosidase activity (Wheeler and Mulcahy, 1989). Therefore, this study focussed on using a biochemical screen in combination with a TILLING approach to select forage sorghum plants that do not synthesize dhurrin by targetting CYP79A1, the enzyme that catalyses the first committed step in dhurrin synthesis. However, as sorghum lines with a high capacity to release HCN may be of commercial use as biofumigants, mutant lines with elevated HCNp were also targetted. Several mutant lines of sorghum with reduced or no potential for HCN production under field conditions were identified. As well as being of direct benefit to agriculture, such mutants provide an important tool for investigating fundamental questions regarding resource allocation to defence-related secondary metabolites and the associated interactions between primary and secondary metabolism.

Results

Generation and screening for cyanogenesis-deficient mutants in *Sorghum bicolor*

To generate a mutant population, 53 000 seeds of an inbred *S. bicolor* line were treated with varying concentrations of ethyl methanesulfonate (EMS) (0.15%–0.4%), resulting in the germination and growth of approximately 16 300 M1 plants. The higher concentrations of EMS affected the fertility of a large proportion of the plants and only approximately 4200 plants set seed, with some panicles producing <5 seeds. Based on seed availability, up to five M2 seed were planted per line, resulting in a population of 5451 individual M2 plants derived from 2709 independent M2 families. Visual assessment of this M2 population growing in the field showed a wide range of variable phenotypes, suggesting that the EMS treatment had been effective (Figure 2). The initial screening of HCNp of 8-week-old plants was conducted in the field using Feigl–Anger (FA) paper (Feigl and Anger, 1966; Takos *et al.*, 2010) and identified 264 putative mutant lines with substantially differing HCNp, either lower or higher, compared with nonmutated parent plants. Following FA paper screening, samples from the youngest fully unfurled leaf were taken from the 264 putative mutant lines at the time points indicated and the HCNp quantified in the laboratory (Figure S1). In several instances, the HCNp determined by FA paper screening was low, whereas the HCNp determined by the quantitative laboratory-based assay, which included the addition of exogenous β-glucosidase, was high. This suggests that a subset of mutant plants may lack endogenous β-glucosidase activity. In this way, 32 putative β-glucosidase mutants were identified (Table 1).

TILLING of the selected 264 plants resulted in the identification of ten putative *CYP79A1* and 13 putative *UGT85B1* mutant lines (Table 1). DNA sequencing showed that the CYP79A1 mutant lines were independently derived and all mutations identified were G:C to A:T transitions (Figure 3 and Table 2), as expected following EMS treatments (Henikoff *et al.*, 2004). Table 2 shows the HCNp in the M2 and M4 generation of a number of the mutant lines. The full effect of the mutation on HCNp would not necessarily be reflected in the M2 generation as these individuals are likely to be heterozygous. Selected mutant plants were allowed to self-pollinate and subsequent generations assayed for HCNp to identify lines homozygous for the identified mutations. The mutant line carrying the stop codon at amino acid 39 and another carrying an A154T amino acid change in the CYP79A1 protein were lost because of poor growth and infertility in the M2 and M3 generation, respectively. The other eight mutant lines appeared healthy under field conditions. Two lines carried silent CYP79A1 mutations, whereas the remaining six carried alteration to the amino acid sequence of CYP79A1 (Figure 3 and Table 2). Line 2-908-1, with the P414L mutation in CYP79A1 showed only a slight reduction in HCNp in the M2 generation (Table 2). However, subsequent generations, homozygous for the mutation, were found to be completely HCNp deficient in all tissue (Table 3) and grew well under field conditions (Figure 4a,b). Sequence analysis of several M4 2-908-1 lines, which show variation in

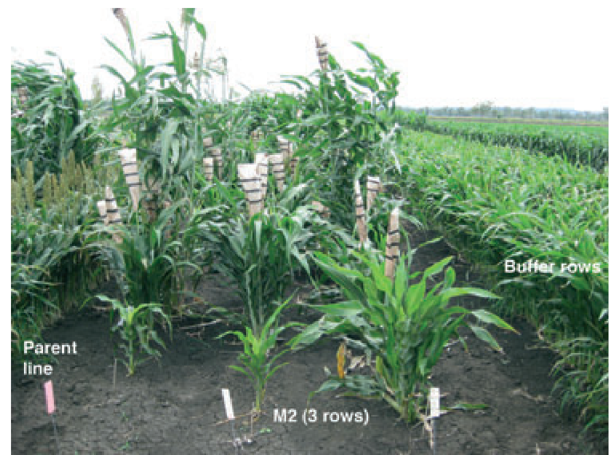

Figure 2 Phenotypic variation observed in the M2 mutant population of sorghum growing in the field in Queensland, Australia. In the centre are three rows of M2 mutant plants, note the variation in height from very short plants in the front to quite tall plants further back. Variations in leaf width and colour were also observed. Flanking the M2 plants are nonmutated parent plants on the left and additional buffer rows on the right. Panicles were bagged to prevent cross-pollination.

Table 1 Classification of *Sorghum bicolor* mutant lines obtained in this study

Gene mutated	Phenotype	No. of families
CYP79A1	Absence or reduction in dhurrin content	9
CYP79A1	Increase in dhurrin content	1
Putative *UGT85B1*	Absence or reduced dhurrin content	13
β-glucosidase 1/2	Devoid of or depleted in β-glucosidase activity	32
Unknown	Regulatory mutant	3
Unknown	Brown mid rib (BMR)	7
Unknown	Increased biomass and/or high sugar	2

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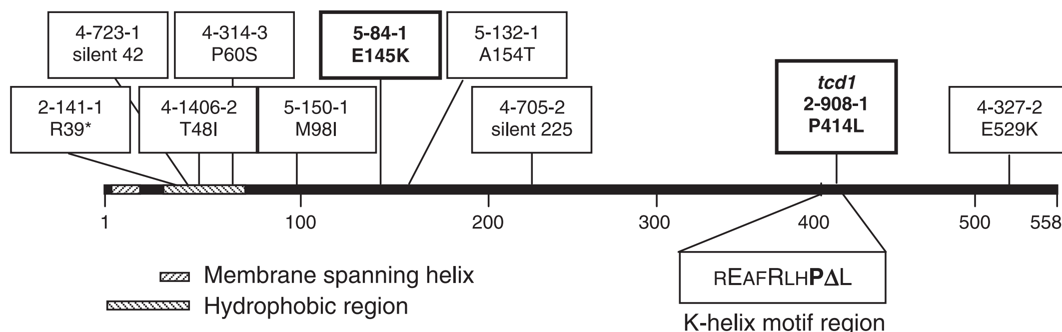

Figure 3 Details and location of the ethyl methanesulfonate-induced mutations identified in CYP79A1. The helix K consensus motif (KETLR, specifically REAFR in sorghum) is located very close to the P414L mutation as shown in the figure.

Table 2 Details of the mutant lines identified to have a mutation in the CYP79A1 gene

			HCNp (mg/g DW)		
			M2 Field grown		M4
Line	nt mutated	Codon change	November 8 ~8 weeks	January 9 ~16 weeks	Greenhouse grown ~5 weeks old
2-141-1	C/T	Stop codon 39	0.24		N/A no seed
4-723-1	C/T	Silent 42		0.43	Not selected
4-1406-2	C/T	T48I		0.11	0.28*
4-314-3	C/T	P60S	0.53		0.18†
5-150-1	G/A	M98I		0.56	Not selected
5-84-1	G/A	E145K		0.97	1.14†
5-132-1	G/A	A154T		0.42	M3 infertile
4-705-2	C/T	Silent 225		0.25	0.31†
2-908-1	C/T	P414L	0.63		<0.03*
4-327-2	G/A	E529K	0.13		0.26†
Control—Av.	–	–	0.71	0.39	0.40

HCNp, hydrogen cyanide potential.

HCNp is higher in younger plants as can be seen from a comparison of the HCNp of the nonmutated parent plants at approximately 8 and 16 weeks. The M4 generation was grown during winter in a greenhouse, which may explain the difference in HCNp observed in the young M2 and M4 control plants.

*Homozygous, confirmed by sequencing.

†Homozygosity not yet confirmed by sequencing.

Table 3 HCNp (mg/g DW) determined in the leaf, sheath and root tissue of adult *tcd1*, *acdc2* and *acdc3* mutants

	Parent line	*tcd1*	*acdc2*	*acdc3*
Leaf	2.09 ± 0.08	0 ± 0.01	0.07 ± 0.03	0.05 ± 0.02
Stem	1.21 ± 0.23	0 ± 0.00	0.36 ± 0.12	0.23 ± 0.03
Root	2.04 ± 0.76	0 ± 0.01	1.04 ± 0.29	1.04 ± 0.64

HCNp, hydrogen cyanide potential.

the HCNp of the different siblings (Figure 5), confirmed that a-cyanogenicity is determined by the presence of the mutation. Lines 2-908-1-1-2, 2-908-1-1-5 and 2-908-1-5-2 are homozygous for the mutation and have negligible HCNp, while the wild-type sibling 2-908-1-3-2 and heterozygous sibling 2-908-1-4-1 have high HCNp (Figure 5). The acyanogenic 2-908-1 mutant line was designated as *tcd1* (totally cyanide deficient 1) and was analysed further.

Three additional independent mutant lines (2-1307-2, 4-565-1 and 4-970-1) identified as having very low HCNp in the leaves of adult plants showed no mutations in the *CYP79A1* and *UGT85B1* structural gene sequences. M5 plants of these three mutant lines grown in the field were healthy with no apparent susceptibility to fungal or insect attack or requirement for additional nitrogen fertilization (Figure 4c–h). The lines grew well, particularly line 2-1307-2, which grew taller and leafier than control plants (Figure 4d,e). While subsequent analysis showed that HCNp in very young leaf tissue (<3-leaf stage; Figure 6a) and etiolated seedlings of these mutants are comparable to the levels in leaves of nonmutated parent lines, the HCNp in these lines drop rapidly to levels below or equivalent to that of the leaves of nonmutated parent plants at the 4-leaf stage (Figure 6a). Quantitative assays in adult M3 and M4 individuals, as well as FA paper assays of adult M5 individuals from each of these lines (Figure 6b), confirm that negligible or very low HCNp is present in mature leaf tissue. However, HCNp of 4-565-1 and 4-970-1 remains high in the sheath and root tissue

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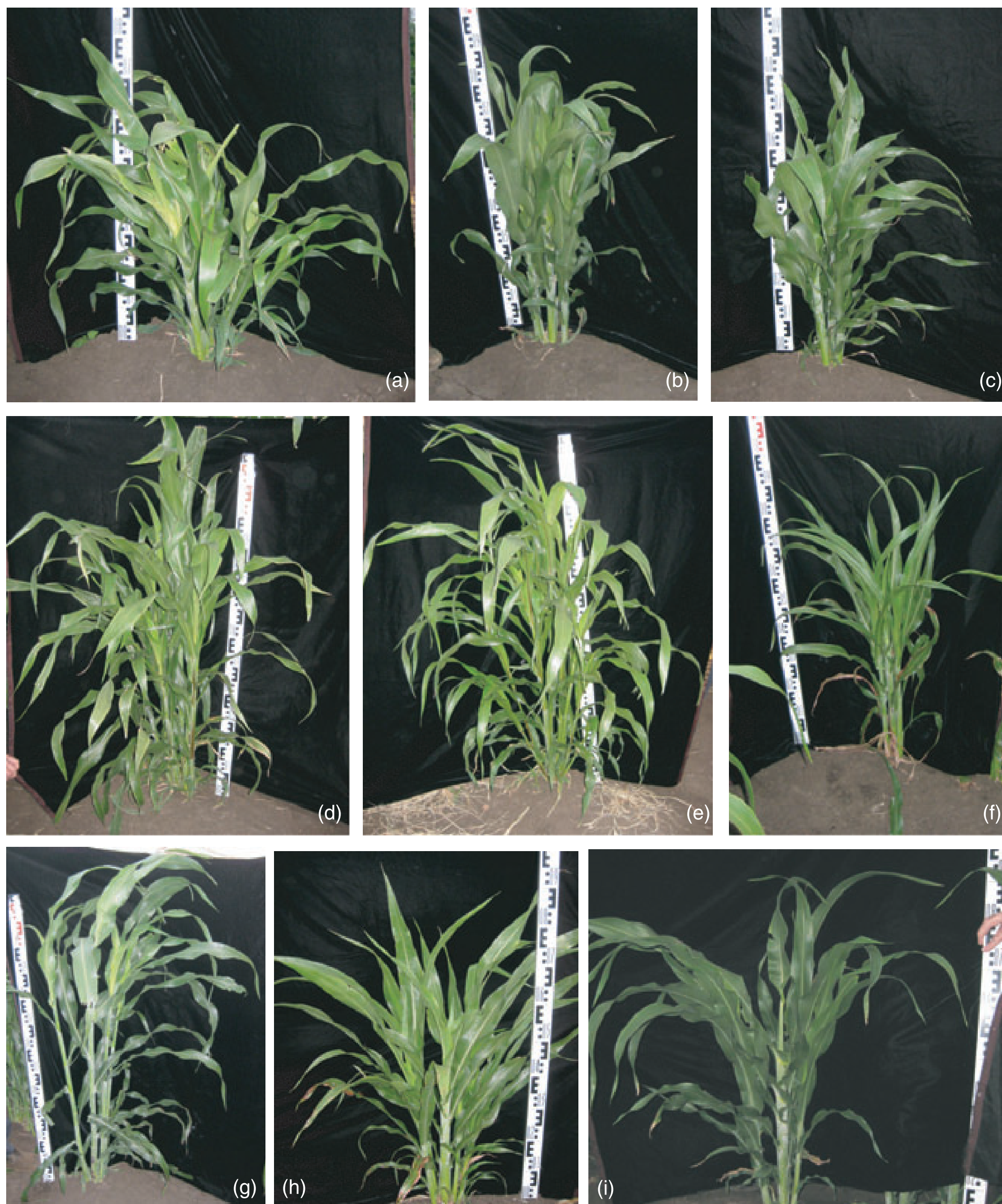

Figure 4 The selected M5 sorghum mutants at approximately 9 weeks of age. (a) acyanogenic *tcd1* individual 2-908-1-1-5 (0.5 m) (b) acyanogenic *tcd1*, 2-908-1-5-2. (0.7 m); (c) *acdc2*, 4-565-1-11-2, (0.6 m); (d) *acdc1*, 2-1307-2-10-1, (0.9 m); (e) *acdc1*, 2-1307-2-10-5, (0.9 m); (f) *acdc3*, 4-970-1-1-2, (0.6 m); (g) *acdc3*, 4-970-1-6-1, (1.0 m); (h) *acdc3*, 4-970-1-10-3, (0.6 m). (i) Nonmutated parent line, (0.6 m). Height stick is marked with 0.1-M increments.

(Table 3). These three lines, 2-1307-2, 4-565-1 and 4-970-1, were designated adult cyanide deficient category (*acdc*) mutants 1-3, respectively.

Analysis of CYP79A1 activity using microsomal preparations

The biochemical activity of CYP79A1 in 4-day-old-etiolated seedlings from the nonmutated parent line and selected *tcd1* and *acdc1-3* mutant lines was measured using microsomal preparations. The germination rate of the seeds from all selected lines was high and the only clear phenotypic effect was observed in the *tcd1* line, which showed slower growth rates at the seedling but not the adult stage. To conserve the valuable seed resource, a procedure was developed to isolate microsomes from as little as approximately 0.5 g etiolated seedling material. Mutant and wild-type seedlings afforded similar yields with approximately 370 μg microsomal protein obtained from 0.5 g of seedling material.

The catalytic activity of CYP79A1 and CYP71E1 requires electron transfer from NADPH-cytochrome P450 oxidoreductase (CPR; Jensen and Møller, 2010). The two P450s as well as CPR are membrane bound and thus recovered in the microsomal preparation. Because the EMS treatment might affect CPR, the CPR activity was measured in wild-type lines and in the individual TILLING lines. The parent line showed an activity of 3.1 nmol cytochrome *c* reduced per min per seedling while the

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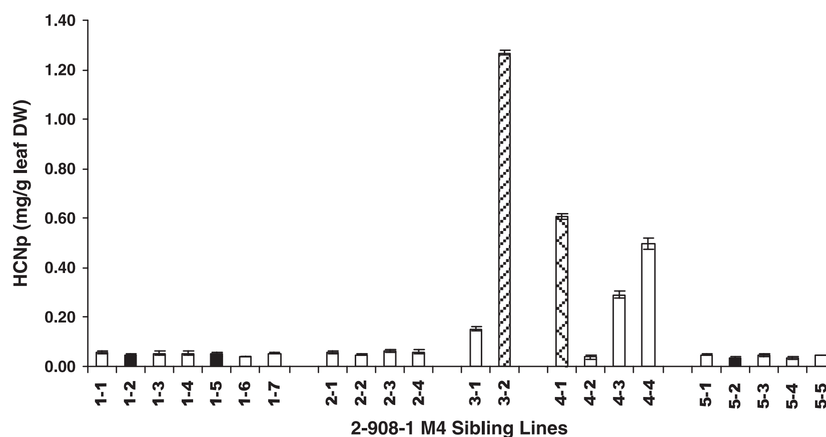

Figure 5 Hydrogen cyanide potential among *tcd1* M4 siblings showing that seedlings homozygous for the P414L mutation are acyanogenic. Solid black, homozygous for the P414L mutation; diagonal grey lines, homozygous for the wild type; hatched pattern, heterozygous for the mutation.

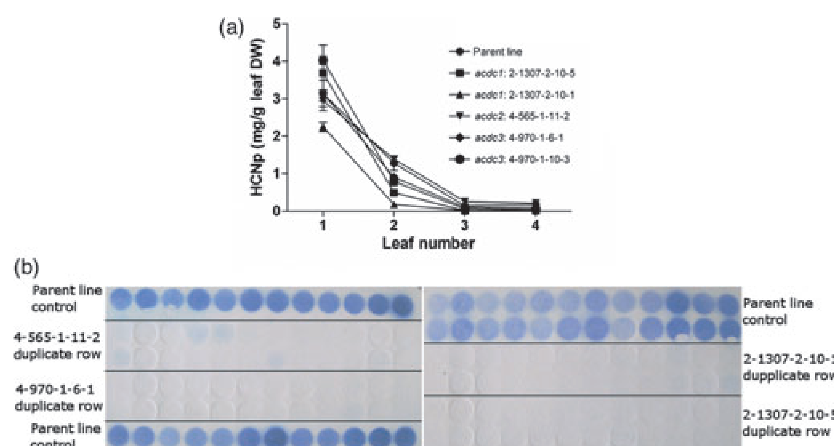

Figure 6 (a) Hydrogen cyanide potential of the leaf tissue of the *acdc1-3* mutant lines during early seedling growth (up to 4-leaf stage). (b) Feigl–Anger test of 15 M5 field-grown adult plants demonstrating that mature leaves of the *acdc* mutant lines are acyanogenic.

activity of the TILLING lines varied in the range from 2.6 to 3.6 nmol cytochrome *c* reduced per min per seedling (Table 4). Thus, none of the TILLING lines showed a substantial reduction in CPR activity compared with nonmutated parent lines.

The CYP79A1 and CYP71E1 activity in the microsomal membranes was monitored by their ability to metabolize radiolabelled tyrosine in the presence of a saturating amount of NADPH. The analysed *tcd1* M4 line was completely devoid of CYP79A1 activity resulting in no metabolism of the administered radiolabelled tyrosine (Figure 7a). At saturating substrate concentrations, the *acdc* mutants showed little or no reduction compared with the parent line (Figure 7a). The *acdc2* line showed only a 20% reduction of activity, while *acdc1* and the three, *acdc3* siblings showed no reduction in CYP79A1 activity. In none of the TILLING lines did *p*-hydroxyphenylacetaldoxime or *p*-hydroxyphenylacetonitrile accumulate, indicating that the activity of the subsequent enzyme in the pathway, CYP71E1, was not limiting for the conversion of intermediates into *p*-hydroxymandelonitrile (Figure 7a). The latter is the final product of the dhurrin pathway obtained when membrane-bound enzymes of microsomal preparations is used as the source.

Table 4 NADPH-cytochrome P450 oxidoreductase activity in microsomal preparations isolated from different sorghum mutants and from the nonmutated parental line

Sorghum line	cyt c reduced (nmol/min per seedling)
Parent line	3.1
tcd1 (2-908-1-1-5)	3.2
tcd1 (2-908-1-5-2)	3.4
acdc1 (2-1307-2-10-1)	3.5
acdc1 (2-1307-2-10-5	3.1
acdc2 (4-565-1-11-2)	2.9
acdc3 (4-970-1-1-2)	3.6
acdc3 (4-970-1-6-1)	2.6
acdc3 (4-970-1-10-3)	3.5

Analysis of the TILLING lines as 3- to 4-day-old etiolated seedlings showed that the *tcd1* mutant does not contain any dhurrin (Figure 7b). This was consistent with the absence of CYP79A1 activity in the microsomal preparations isolated from the same lines. Dhurrin content of the seedlings from the other mutant lines showed no direct correlation with the measured activity of CYP79A1. In wild-type seedlings, the high dhurrin content of dark grown seedlings decreases rapidly after approximately 4 days and the amount of dhurrin presence reflects the balance between rate of synthesis and turn-over. Hence, a direct relationship between CYP79A1 activity and dhurrin content is not necessarily expected.

CYP79A1 and CYP71E1 content of the sorghum TILLING lines as determined by immunoblotting

Antibodies were raised against different specific surface-exposed peptide sequences of CYP79A1 and CYP71E1. Western

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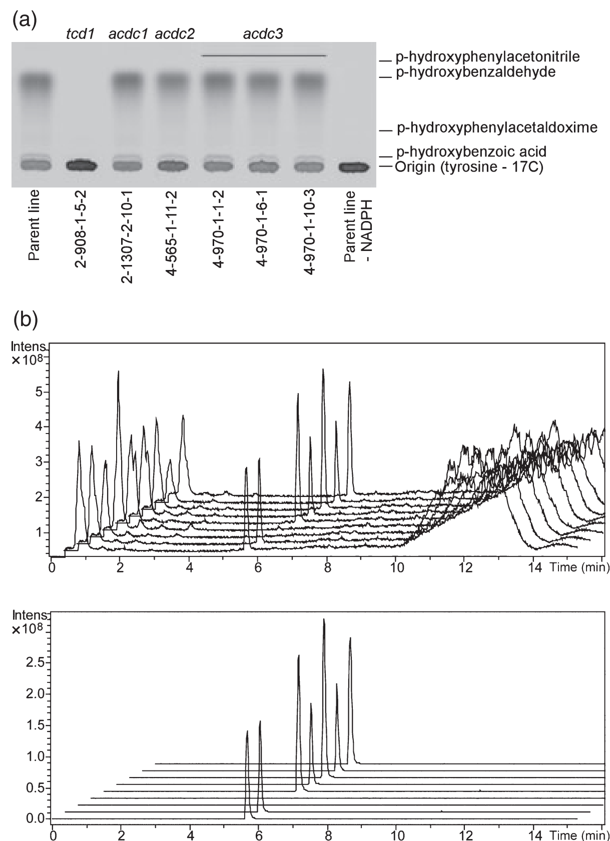

Figure 7 Assessment of CYP79A1 activity in microsomal preparations from nonmutated parent lines and selected TILLING mutants. (a) CYP79A1 and CYP71E1 activity measured by administration of radio-labelled tyrosine to the microsomal system. No accumulation of *p*-hydroxyphenylacetaldoxime is observed demonstrating that all lines possess active CYP71E1. *p*-Hydroxymandelonitrile is labile and is therefore monitored as *p*-hydroxybenzaldehyde. (b) LC-MS analysis of the dhurrin content (RT 5.7 min) shown as total ion traces (top panel) and by specific ion monitoring (lower panel). Traces shown from front to back are two nonmutated parent lines, two *tcd1* siblings, followed by *acdc* mutants.

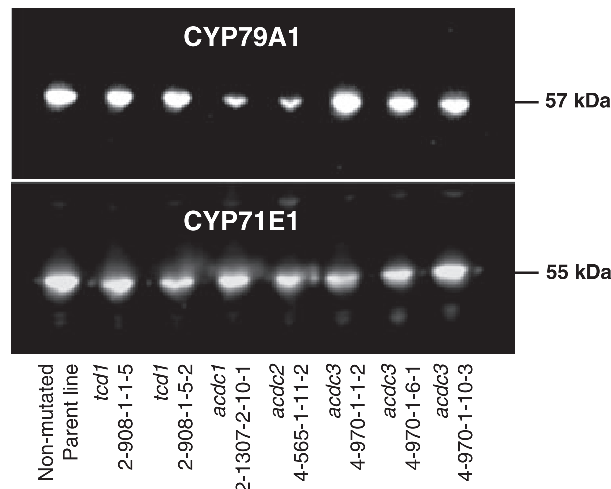

Figure 8 Western blot analysis of CYP79A1 and CYP71E1 in microsomal preparations from the nonmutated parent line and mutant lines.

blot analysis of the CYP79A1 and CYP71E1 content of microsomal preparations isolated from the parent line and from different TILLING lines are shown in Figure 8. CYP79A1 and CYP71E1 proteins were shown to be present in all mutant lines and the content of CYP79A1 and CYP71E1 per seedling did not vary substantially as determined by a semi-quantitative Western blot analysis (Figure 8).

Molecular modelling of mutations in CYP79A1

To gain an understanding of the possible effect of the identified mutations on CYP79A1 activity, a homology model of CYP79A1 was made based on the solved crystal structures of relevant P450s (Figure 9a; Jensen *et al.*, 2011). Two of the mutations (E145K, A154T) are positioned in the proposed substrate-binding site and would directly alter the shape and charge distribution within the tyrosine substrate-binding pocket. The HCNp in both M2 and M4 plants in line 5-84-1 (E145K) was higher than control plants, suggesting that alteration of the negatively charged E residue to a positively charged K residue at position 145 may have the potential to increase substrate affinity. In agreement with this hypothesis, this residue is modelled to interact with the partly negatively charged *p*-hydroxy group of the tyrosine substrate, and the presence of a positively charged K residue in the mutant compared with the negatively charged E residue in the wild-type enzyme may indeed serve to increase substrate affinity. The P414L mutation in *tcd1* is found 10.7 Å away from the substrate-binding site, and consequently, this mutation would not directly interfere with binding of substrate (Figure 9b). However, the P414L mutation is located at the end of helix K (consensus sequence, KETLR), and replacement of this highly conserved P residue (Paquette *et al.*, 2009) with an L so close to this motif is likely to affect enzyme activity. The arginine (R) in the PERF motif (a P450 signature sequence), forms a salt bridge with the glutamic acid (E) and arginine (R) residues in the KETLR motif, and this E–R–R triad is generally thought to lock the haem pocket of the active site into the proper position and to stabilize the overall P450 core structure (Hasemann *et al.*, 1995). The P414L mutation is likely to obstruct this structural organization resulting in overall destabilization and loss of catalytic properties. Two mutations (M98I and E529K) are on the periphery of the enzyme and the enzyme's apparent inability to metabolize tyrosine to *p*-hydroxyphenylacetaldoxime is not easily explained in a structural context. Three additional mutations identified in the screen (R39stop, T48I and P60S) are located in the transmembrane anchor. The transmembrane anchor was not included in the homology model because of the lack of available templates.

Discussion

In this study, a combined biochemical screen and TILLING approach has been used successfully to identify sorghum plants in which cyanogenesis has been substantially altered without using transgenic approaches. We have produced several viable lines, including acyanogenic or low HCNp lines as well as lines that accumulate high HCNp in the adult stages. In addition to being of agronomic value, these lines provide an excellent resource for increasing our understanding of the molecular mechanisms involved in cyanogenesis and factors that affect its regulation. The TILLING population was screened for mutations in two of the three key biosynthetic genes in the cyanogenesis

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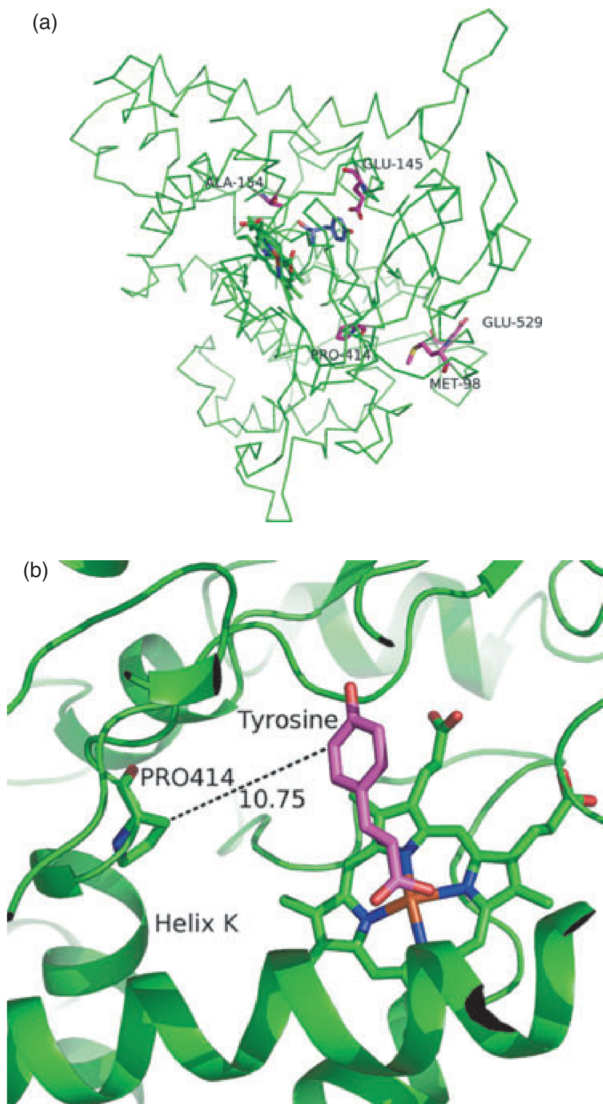

Figure 9 Homology modelling of CYP79A1 based on solved crystal structures of relevant P450s. (a) Molecular model showing the location of five of the identified mutations in CYP79A1. (b) Specific location and details of the P414L mutation in *tcd1*.

pathway, *CYP79A1* and *UGT85B1*. Ten lines that contain mutations in *CYP79A1* were identified. The majority of these lines exhibit lower or negligible HCNp compared with nonmutated parent lines. In contrast, the line 5-84-1 containing the E145K mutation has considerably higher dhurrin content compared with the nonmutant parent lines and will be the subject of further investigations to determine its value as a biofumigant. Analyses of the lines carrying mutations in *UGT85B1* are ongoing. The dhurrin-producing enzymes from sorghum have been expressed in noncyanogenic plants, either individually or in combination (Kristensen *et al.,* 2005), and the results obtained imply that a nonfunctional CYP71E1 protein would result in the accumulation of reactive *p*-hydroxyphenylacetaldoxime (Møller, 2010b). Thus, screening for mutations in *CYP71E1* was not performed as such plants, if viable, would be likely to have reduced fitness (Bak *et al.*, 2006; Morant *et al.*, 2007).

Acyanogenic sorghum has not been detected in natural populations. In some other species, cyanogenic glucosides are synthesized in the shoot and transported to the root and may act as a nitrogen source for root development (Selmar *et al.*, 1988; Jørgensen *et al.*, 2005a). In transgenic cassava plants, created using RNA interference (RNAi) to target CYP79D1 and D2, even a low rate of cyanogenic synthesis in leaves results in transport of substantial amounts of cyanogenic glucoside into tubers. However, in some of the RNAi cassava plants with essentially acyanogenic leaves and a 92% reduction in cyanogenic glucosides in tubers, normal roots and tubers still formed when the plants were grown in soil (Jørgensen *et al.*, 2005a).

The rate of dhurrin synthesis in sorghum is normally high during the first few days post-germination after which rates of degradation exceed those of synthesis (Halkier and Møller, 1989). Catabolism of dhurrin in these early stages of seedling growth leads to the production of ammonia and 4-hydroxyphenylacetic acid (Jenrich *et al.*, 2007). The latter compound, together with other hydroxy- or methoxy-derivatives of phenylacetic acid, was shown to have growth-promoting properties consistent with a role as weak auxins in classical tissue culture experiments (Lee and Skoog, 1965). The observation that *tdc*1 mutants lack dhurrin and show slightly retarded rates of growth when very young may indicate that this cyanogenic glycoside is an important source of growth regulatory metabolites at this critical stage of growth in sorghum. Further experiments to test this hypothesis are underway.

The *tcd1* mutant produces no dhurrin in either the leaves (Figure 7) or the sheath and roots (Table 3). However, growth characteristics of this acyanogenic sorghum *tcd1* mutant, which produces a nonfunctional CYP79A1 protein, is yet to be determined in detail. Under favourable conditions, the growth of the plant is largely unaffected, exhibiting only slightly slower growth in early seedling stages and despite the unusually wet growth conditions experienced during the growing season in 2010 and 2011 in S.E. Queensland, the field grown M5 *tcd1* mutant plants did not show altered susceptibility to insect or fungal attack. Nor do the *tcd1* mutants require additional nitrogen for normal growth and development, as they appear phenotypically similar to parental plants.

The *acdc* mutant lines have a high HCNp in young seedlings and possess a microsomal enzyme system with activity comparable to that of nonmutated parental seedlings yet have a much reduced HCNp in mature leaf tissue compared with control plants. Analysis of the ontogeny of HCNp from seedling stage up to the 4-leaf stage shows that the HCNp drops rapidly in *acdc1* and *acdc3* mutant lines (Figure 6a). The HCNp decrease in *acdc2* is less rapid, but additional experiments demonstrated that the residual HCNp is restricted to the leaf sheath tissue (Table 3) and, like *acdc1* and *acdc3* mutants, is not found in leaf tissue.

Previous studies (Busk and Møller, 2002) indicate that dhurrin biosynthesis in adult plants occurs mainly in the sheath and is upregulated at the transcriptional level (*CYP79A1* and *CYP71E1*) by the addition of exogenous nitrogen. While the *CYP79A1* and *CYP71E1* transcripts in the leaf are easily detectable in the young highly cyanogenic control plants, the transcripts are not detectable by northern analysis in adult leaf tissue in either nonmutated parent plants or any of the mutant lines examined when grown under normal conditions (data not shown). The transcriptional response to increased exogenous nitrogen availability in adult mutant plants is yet to be determined. The Western blot analysis shows the protein levels of CYP79A1 and

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CYP71E1 are not altered substantially in the microsomal preparations from young mutant seedlings including the acyanogenic *tcd1* mutant. This suggests the lack of dhurrin production in the *tcd1* mutant has not triggered a compensatory feedback mechanism that is detectable at the protein level.

Dhurrin mobilization and transport in sorghum is not well understood. While the *acdc* mutants produce shoot tissue that is essentially acyanogenic at adult stages, there is no substantial reduction in the dhurrin content in the roots of adult plants (Table 3) compared with levels found in nonmutated parent plants. This may indicate that root accumulation is not dependent on biosynthetic capacity in leaves. While sheath tissue has been identified as the primary site of synthesis in older plants (Busk and Møller, 2002), low levels of *CYP79A1* transcripts have also been observed in sorghum roots (Natalie O' Donnell, unpubl. data), suggesting some synthesis may normally occur in the roots and that this is unaffected in the *acdc* mutants. In cassava, root tissue has also been reported to be able to carry out *de novo* synthesis of cyanogenic glucosides (Du *et al.*, 1995). The low dhurrin levels in the adult leaves of *acdc* mutants could result from mutations in leaf-specific regulatory genes controlling the expression levels of biosynthetic genes and/or degradation pathways and/or alternatively mutations may affect dhurrin transport or storage in adult plants. Because the dhurrin levels are essentially negligible in the leaves and very low in the sheath of these adult plants, a mutation in dhurrin transport seems less likely.

When grown in the field, the *acdc* mutants, as well as the *tcd1* mutant, are as tall or taller than nonmutated parent plants and are leafier. This supports the notion that the lack of dhurrin accumulation does not impede plant growth. The growth/defence nexus assumes that the cost of synthesis of bioactive defence compounds necessitates a trade off between growth and defence (Endara and Coley, 2011). The possible presence of several mutations unrelated to cyanogenesis in the *acdc* mutant plants means that a direct link between low dhurrin accumulation and enhanced growth cannot be made at this stage, but it is noteworthy that the link between the absence of dhurrin in the leaves of adult plants and tall, bushy growth was observed in all three independent *acdc* lines. A similar phenomenon was observed in white clover (Kakes, 1989).

Dhurrin production in sorghum control populations is high during initial germination and seedling growth and then declines as the plant matures (Loyd and Gray, 1970; Wheeler *et al.*, 1990; Busk and Møller, 2002; Møller and Conn, 1980) making it suitable as a highly nutritious forage crop. However, environmental factors, such as drought and high nitrogen, are problematic as they may increase the dhurrin content in adult sorghum plants to toxic levels (Loyd and Gray, 1970; Wheeler *et al.*, 1990). Results from a preliminary drought experiment indicates that dhurrin production is not induced by drought in the shoot tissue of adult *acdc* plants and suggests that dhurrin synthesis or breakdown may be controlled by different developmental and environmental-stress regulatory pathways. The availability of the *acdc* mutants may assist in dissecting the molecular pathways regulating cyanogenesis in response to developmental and environmental signals.

The retention of cyanogenic glucosides throughout young *acdc* mutant seedlings and in the roots of adult plants retains much of the potential protective capacity of cyanogenesis towards crop pests. There are a number of serious fungal diseases in sorghum (Gupta and Paul, 2002). Recent reports suggest that the stoichiometric release of HCN during fungal-induced ethylene production in plants helps retard fungal growth independently of the ethylene-induced responses (Seo *et al.*, 2011). ß-Glucosidase and hydroxynitrile lyase–mediated release of HCN from dhurrin stored in the vacuole following cellular disruption and CYP79A1-catalysed oxime release induced by the oxygen burst associated with the hypersensitive response may potentially inhibit the growth of pathogenic fungi (Møller, 2010a). However, rubber tree (*Hevea brasiliensis*) varieties susceptible to the fungus South American Leaf Blight had a high HCNp, while resistant varieties had low HCNp (Lieberei, 2007). Interestingly, some of the high HCNp mutants identified in the sorghum TILLING population appeared more susceptible to fungal attack than low HCNp mutants. The susceptibility could result from specialised fungal pathogens ability to detoxify HCN (Møller, 2010b). Trials are underway to test the fitness of the selected mutant plants in the field.

To benefit the agricultural industry, the desired characteristic of these forage sorghum plants, lacking the capacity to produce dhurrin, now needs to be introgressed into commercial elite hybrids. Utilization of the mutated *CYP79A1* gene in hybrids will involve a process of crossing the elite mutants with appropriate commercial male and female forage sorghum parent lines. The development of each new parent will involve pedigree crossing and assaying individual plants in the F_2 generation for the absence of HCNp. In addition, selection for broader morphological characteristics will be required.

Experimental procedures

Plant material

The mutant population was generated using a near-isogenic *S. bicolor* (L.) Moench inbred parent line (Pacific Seeds, Toowoomba, Qld, Australia). Seeds were treated with 0.15%–0.4% EMS for 16 h in batches of approximately 1000 seed/500 mL with shaking (approximately 50 r.p.m.), then rinsed thoroughly with water. A total of 53 000 treated seeds were sown in the field at the Pacific Seeds Research Station (Gatton, Queensland). The site was prepared by deep ripping the paddock twice, 3–4 months before planting and spraying with Roundup (Monsanto, http://www.monsanto.com.au). A complete fertilizer [120 kg/ha of Urea (46% N) and 120 kg/ha of CK-88 (15.1% N; 4.4% P; 11.5% K; 13.6% S)] was applied 6 weeks before planting and additional nitrogen (40 kg/ha urea) supplied 5 weeks postplanting. Weeds were controlled with Atrazine (3 L/ha; Syngenta, http://www.atrazine.com) before planting and again after 4 weeks (1 L/ha) and with inter-row cultivation.

To ensure self-pollination, each panicle was bagged prior to anthesis. Panicles were harvested and threshed individually, resulting in 4185 individual M2 selections. Up to five seeds from each M2 line were sown in the field, resulting in 5451 individual M2 plants derived from 2709 independent M2 lines. These plants were screened phenotypically for cyanide levels using Feigl–Anger (FA) paper when approximately 8 weeks old. The main panicle of all M2 plants was bagged and threshed individually. Selected M3 mutant lines were sown in the field, whereas lines of the M4 generation were planted in a greenhouse at Pacific Seeds (Toowoomba) to facilitate growth over the winter. The main panicle for all plants of the

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M3 and M4 generations were also bagged to allow generation of homozygous mutants. All seed was stored or used for propagation purposes. Numbering of individual mutant lines allows the identification of the ancestry of each plant and easy tracking through every generation. For example, in 2-908-1-1-5, the first number represents the second EMS seed treatment batch, the 908 is the M1 reference number and each subsequent number identifies the plant selected in the following generations.

Assays of the cyanide content of mutant lines

Feigl–Anger (FA) papers (Feigl and Anger, 1966; Miller *et al.*, 2006) were used to identify mutants with altered HCNp in all M2 plants (approximately 6100) (Gleadow *et al.*, 2010; Takos *et al.*, 2010). Three leaf discs (approximately 0.5 cm diameter) were taken from the youngest fully unfurled leaf of 8-week-old plants and placed in a single well of a 96-well plate, covered with a sheet of FA paper and sealed. Every plate included samples from eight nonmutated parent plants as controls. The sealed plate was frozen and thawed to lyse the cells and incubated at 25 °C for approximately 30 min, allowing HCN to evolve and form a concentration-dependent blue spot above each well (Miller *et al.*, 2006; Takos *et al.*, 2010). FA papers were photographed as a permanent record.

Based on the FA paper screening, 264 individual M2 plants showing substantial differences (either low or high) in HCNp compared with nonmutated parent plants were selected for quantitative HCNp analysis using a colorimetric method (Gleadow *et al.*, 2010). The youngest fully unfurled leaf was sampled from approximately half of the M2 plants at approximately 8 weeks old, while the rest were sampled at approximately 16 weeks. Previous experiments have shown that dhurrin can be quantified accurately from dried material (Haskins *et al.*, 1984). Therefore, dried, ground tissue (approximately 10 mg) was placed in a vial containing 300 μL of 0.1 M citrate buffer (pH 5.5) with 1.12 units/mL β-D-glucoside glucohydrolase (EC 3.2.1.21). A PCR tube containing 200 μL of 1 M NaOH was placed in the vial, which was then sealed and incubated (1 h at RT followed by 15 h, at 37 °C). The NaCN content of the NaOH trap was quantified colorimetrically using NaCN as the standard (Gleadow *et al.*, 2010).

Genomic DNA extraction

Genomic DNA from the selected 264 M2 plants and nonmutated parent plants was isolated using the MagAttract DNA extraction kit (Qiagen, http://www.qiagen.com). Multiple plates, containing 100 ng DNA aliquots in individual wells were prepared to enable the analysis by PCR and TILLING using primers specific to *CYP79A1* and *UGT85B1*.

Detection of mutations in the target genes

Nested PCR with M13 tailed primers (Table S1) was used to amplify the 5′ and 3′ region of *CYP79A1* and *UGT85B1* genes (Genebank accession numbers U32624 and AF199453, respectively). All PCR reaction mixtures (20 μL) contained 0.5 U *Pfu* DNA polymerase (Promega, http://www.promega.com) in buffer containing 2 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 μM gene specific forward and reverse primers. Following the first PCR, the products were pooled threefold in a matrix designed to allow identification of individuals, and re-amplified using a combination of 0.2 μM M13 tailed gene specific nested primers and 1 μM of the corresponding forward and reverse M13 primers fluorescently labelled with IR dye 700 and 800 nm, respectively (Eurofins MWG operon, http://www.eurofinsdna.com), to allow visualization following gel separation on the LiCor 4200 (Table S1). The thermocycling conditions are detailed in Table S1.

Following the second PCR, the amplified products were denatured and re-annealed (99 °C—10 min; 70 °C—20 s, −0.3 °C/cycle × 70 cycles; 48 °C—20 s, −0.3 °C/cycle × 50; 4 °C hold) to form heteroduplex DNA. Mutations were detected by digestion of the heteroduplexed DNA using CEL1 enzyme (Oleykowski *et al.*, 1998). Cleaved products were visualized by electrophoresis on the Li-Cor 4200 DNA Analyser (Li-Cor Bioscience, http://biosupport.licor.com). GelBuddy (http://www.gelbuddy.org; Zerr and Henikoff, 2005) was used to analyse the gels and identify the mutations in the M2 individual.

Sequencing of cyanide-deficient mutants

The CYP79A1 gene PCR products were amplified from mutant genomic DNA and purified using the Promega Wizard kit (Promega, http://www.promega.com) with an added final ethanol precipitation step to optimize DNA purity and sequenced using the Applied Biosystems PRISM BigDye Terminator Mix(Applied Biosystems, http://www.appliedbiosystems.com.au/).

Preparation of microsomes

Seventy seeds of *S. bicolor* parent lines and of selected TILLING lines were germinated in fine grade vermiculite in the dark (4 days at 23 °C). Microsomes were isolated from approximately 0.5 g of seedling material. Each class of seedlings was harvested separately, counted to correct for small differences in germination percentage, weighed and ground in a chilled mortar with 600 mg polyvinylpolypyrrolidone in 8 mL isolation buffer [250 mM sucrose, 100 mM Tricine (pH 7.9), 50 mM NaCl, 2 mM DTT]. The homogenate was filtered through a nylon cloth (22 μm mesh) and centrifuged (10 min, 10 000 ***g***). Supernatant was centrifuged (1 h, 100 000 ***g***) and the microsomal pellet resuspended in 8 mL isolation buffer using a tiny marten paintbrush to wash away soluble substrates and cofactors bound to the microsomal membranes. Following re-centrifugation as described earlier, the microsomal pellet was resuspended in 120 μL resuspension buffer [50 mM (pH 7.9), 20 mM NaCl, 2 mM DTT] and final volume was adjusted so that 3.6 μL of the microsomal extract corresponded to the amount of microsomal protein isolated from a single seedling.

Analysis of NADPH-cytochrome P450 oxidoreductase activity

The activity of NADPH-cytochrome P450 oxidoreductase (CPR) in each of the microsomal preparations was measured by its ability to reduce cytochrome *c* (Horseheart cytochrome *c*, Sigma, cat no 7752; http://www.sigmaaldrich.com) as monitored spectrometrically at 550 nm. The sample cuvette (1 mL) contained 50 μL 1 mM cytochrome *c* and 5 μL microsomal membrane preparation in 50 mM KP_i (pH 7.9). Following monitoring of the background reduction rate for 3 min, 10 μL of 62 mM NADPH was added and the initial rate of cytochrome *c* reduction was measured. No NADPH was added to the reference cuvette. In the assay conditions used, 1 nmol of CPR will reduce about 3000 nmol of cytochrome *c* per min enabling quantification of the activity in each sorghum seedling (Guengerich *et al.*, 2009).

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Analysis of CYP79A1 activity

The activity of CYP79A1 in the microsomal membrane preparations was assayed in 20.2-μL reaction mixtures containing 2.5 μL L-[UL-^{14}C]-tyrosine (482 mCi/mmol), 2.5 μL 8 mM NADPH and 5 μL resuspension buffer. The reaction was initiated by addition of 7.2 μL microsomes. Following the incubation period (30 min, 30 °C), the entire reaction mixture was applied to silica gel 60F254 TLC plates (Merck, http://www.merck.dk). Following development in ethyl acetate: toluene (1 : 5 v/v), the formation of radiolabelled dhurrin pathway intermediates was monitored and quantified by phosphor imaging (Typhoon; GE Healthcare, http://www.gelifesciences.com). In a parallel set of experiments, the incubation mixtures were fortified with 2.5 μL 1 mM L-tyrosine to saturate the enzyme system with substrate over the entire incubation period. The position of the different intermediates on the TLC plates was determined by co-application of unlabelled reference compounds and monitored by UV absorption.

Quantification of dhurrin content in dark grown seedlings

Ten 4-day-old dark grown seedlings of the nonmutated parent line and each of the four selected mutant TILLING lines were weighed and homogenized in 600 μL 85% MeOH in a microcentrifuge tube using a pointed pestle. Following extraction (4 h, RT, gentle shaking), the homogenate was centrifuged (10 min, 3000 ***g***). Aliquots (20 μL) were diluted with 60 μL H_2O and filtered through a membrane by centrifugation. Dhurrin content was determined by injection of 0.1 and 2 μL aliquots in the LC-MS and analysed as outlined later.

LC-MS experimental data

Analytical LC-MS was carried out using an Agilent 1100 Series LC (Agilent Technologies, http://www.home.agilent.com) hyphenated to a Bruker HCT-Ultra ion trap mass spectrometer (Bruker Daltonics, http://www.bdal.com/). A Zorbax SB-C18 column (Agilent; 1.8 μM, 2.1 × 50 mm) was used at a flow rate of 0.2 mL/min, and the oven temperature was maintained at 35°. The mobile phases were the following: A, water with 0.1% (v/v) HCOOH and 50 μM NaCl; B, acetonitrile with 0.1% (v/v) HCOOH. The following gradient program was used: 0–0.5 min, isocratic 2% B; 0.5–7.5 min, linear gradient 2%–40% B; 7.5–8.5 min, linear gradient 40%–90% B; 8.5–11.5 isocratic 90% B; 11.60–17 min, isocratic 2% B. The flow rate was increased to 0.3 mL/min in the interval 11.2–13.5 min. The mass spectrometer was run in positive electrospray mode. Data was analysed using the Bruker Daltonics Data Analysis software with comparison to dhurrin standards.

Immunoblotting

CYP79A1 specific antibodies were obtained following immunization of rabbits using a 14-residue peptide, AGVEAVDLSESKSD (residues 520–533). In a similar manner, specific antibodies towards CYP71E1 were obtained using the 12-mer peptide, VVPTKYKNRRAA (residues 520–531). Microsomal preparations (2.4 μL) corresponding to the amount of microsomal protein isolated from 0.67 seedlings were electrophoresed on a 12% SDS-PAGE gels in MOPS buffer for 1 h at 200 V. The separated proteins were transferred to nitrocellulose membranes (45 min at 100 V) and the membranes blocked (1 h at room temperature) in 5% skim milk powder solution (PBS-T), washed and the immunoreactions carried out in 5% skim milk powder solution (PBS-T) using either antibodies to CYP79A1 at 1 : 2000 dilution or to CYP71E1 at 1 : 5000 dilution (1 h at room temperature). The blot was incubated with secondary antibody for 1 h at room temperature at a dilution of 1 : 2000.

Homology modelling of mutations affecting CYP79A1

The protein model of CYP79A1 was built using the Orchestra protein modelling component of the Sybyl software (Tripos, http://tripos.com/) using coordinates for the solved crystal structures of relevant CYPs (Jensen *et al.*, 2011). Structural models and position of mutations were visualized using PYMOL (http://www.pymol.org). APBS was used for the calculation and visualization of the electrostatic potential (Baker *et al.*, 2001). The final structural model included 489 residues thus missing the N-terminal residues 1–69.

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Author contributions

The project was initiated by RMG; strategic approach and experimental planning by ADN, RMG, JDH, BLM and CKB. All authors contributed to experimental and/or field work. All authors provided intellectual input into the writing and preparation of this manuscript.

References

Akazawa, T., Miljanich, P. and Conn, E.E. (1960) Studies on cyanogenic glycoside of *Sorghum vulgare*. *Plant Physiol.* **35**, 535–538.

Bak, S., Kahn, R.A., Nielsen, H.L., Møller, B.L. and Halkier, B.A. (1998) Cloning of three A-type cytochromes P450, CYP71E1, CYP98, and CYP99 from *Sorghum bicolor* (L.) Moench by a PCR approach and identification by expression in *Escherichia coli* of CYP71E1 as a multifunctional cytochrome P450 in the biosynthesis of the cyanogenic glucoside dhurrin. *Plant Mol. Biol.* **36**, 393–405.

Bak, S., Paquette, S.M., Morant, M., Morant, A.V., Saito, S., Bjarnholt, N., Zagrobelny, M., Jørgensen, K., Osmani, S., Simonsen, H.T., Pérez, R.S., Heeswijck, T.B.v., Jørgensen, B. and Møller, B.L. (2006) Cyanogenic glycosides: a case study for evolution and application of cytochromes P450. *Phytochem. Rev.* **5**, 309–329.

Baker, N.A., Sept, D., Joseph, S., Holst, M.J. and McCammon, J.A. (2001) Electrostatics of nanosystems: application to microtubules and the ribosome. *Proc. Natl Acad. Sci. USA*, **98**, 10037–10041.

Busk, P.K. and Møller, B.L. (2002) Dhurrin synthesis in sorghum is regulated at the transcriptional level and induced by nitrogen fertilization in older plants. *Plant Physiol.* **129**, 1222–1231.

Du, L., Bokanga, M., Møller, B.L. and Halkier, B.A. (1995) The biosynthesis of cyanogenic glucosides in roots of cassava. *Phytochemistry*, **39**, 323–326.

© 2011 The Authors

Duncan, R.R. (1996) Breeding and improvement of forage sorghums for the tropics. *Adv. Agron.* **57**, 161–185.

Endara, M.-J. and Coley, P.D. (2011) The resource availability hypothesis revisited: a meta-analysis. *Funct. Ecol.* **25**, 389–398.

Feigl, F. and Anger, V. (1966) Replacement of benzidine by copper ethylacetoacetate and tetra base as spot-test reagent for hydrogen cyanide and cyanogen. *Analyst*, **91**, 282–284.

Forslund, K., Morant, M., Jorgensen, B., Olsen, C.E., Asamizu, E., Sato, S., Tabata, S. and Bak, S. (2004) Biosynthesis of the nitrile glucosides rhodiocyanoside A and D and the cyanogenic glucosides lotaustralin and linamarin in *Lotus japonicus*. *Plant Physiol.* **135**, 71–84.

Ghannoum, O. (2009) C4 photosynthesis and water stress. *Ann. Bot.* **103**, 635–644.

Gleadow, R. and Woodrow, I. (2000) Temporal and spatial variation in cyanogenic glycosides in *Eucalyptus cladocalyx*. *Tree Physiol.* **20**, 591–598.

Gleadow, R. and Woodrow, I. (2002a) Constraints on the effectiveness of cyanogenic glycosides in herbivore defence [mini review]. *J. Chem. Ecol.* **28**, 1301–1313.

Gleadow, R. and Woodrow, I. (2002b) Defence chemistry of *Eucalyptus cladocalyx* seedlings is affected by water supply. *Tree Physiol.* **22**, 939–945.

Gleadow, R.M., Vecchies, A.C. and Woodrow, I.E. (2003) Cyanogenic *Eucalyptus nobilis* is polymorphic for both prunasin and specific ß-glucosidases. *Phytochemistry*, **63**, 699–704.

Gleadow, R., Bjarnholt, N., Jørgensen, K., Fox, J. and Miller, R. (2010) Detection, identification and quantitative measurement of cyanogenic glycosides. In *Research Methods in Plant Science: Soil Allelochemicals* (Narwal, S.S., Szajdak, L. and Sampietro, D.A., eds), Chapter 12: pp. 283–310. New Delhi, India: International Allelopathy Foundation, Studium Press.

Gregory, P.J., Ingram, J.S. and Brklacich, M. (2005) Climate and Food Security. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **360**, 2139–2148.

Guengerich, F.P., Martin, M.V., Sohl, C.D. and Cheng, Q. (2009) Measurement of cytochrome P450 and NADPH-cytochrome P450 reductase. *Nat. Protoc.* **4**, 1245–1251.

Gupta, V.K. and Paul, Y.S. (2002) Fungal diseases of pearl millet and sorghum. In *Diseases of Field Crops* (Gupta, V.K. and Paul, Y.S., eds), pp. 128–154, New Delhi: Indus Publishing company.

Halkier, B.A. and Møller, B.L. (1989) Biosynthesis of the cyanogenic glucoside dhurrin in seedlings of *Sorghum bicolor* (L.) Moench and partial purification of the enzyme system involved. *Plant Physiol.* **90**, 1552–1559.

Hasemann, C.A., Kurumbail, R.G., Boddupalli, S.S., Peterson, J.A. and Deisenhofer, J. (1995) Structure and function of cytochromes P450: a comparative analysis of three crystal structures. *Structure*, **3**, 41–62.

Haskins, F.A. and Gorz, H.J. (1986) Relationship between contents of leucoanthocyanidin and dhurrin in sorghum leaves. *Theor. Appl. Genet.* **73**, 2–3.

Haskins, F.A., Gorz, H.J., Hill, R.M. and Youngquist, J.B. (1984) Influence of sample treatment on apparent hydrocyanic acid potential of sorghum leaf tissue. *Crop Sci.* **24**, 1158–1163.

Henikoff, S., Till, B.J. and Comai, L. (2004) TILLING. Traditional mutagenesis meets functional genomics. *Plant Physiol.* **135**, 630–636.

Jenrich, R., Trompetter, I., Bak, S., Olsen, C.E., Møller, B.L. and Piotrowski, M. (2007) Evolution of heteromeric nitrilase complexes in Poaceae with new functions in nitrile metabolism. *Proc. Natl Acad. Sci. USA*, **104**, 18848–18853.

Jensen, K. and Møller, B.L. (2010) Plant NADPH-cytochrome P450 oxidoreductases. *Phytochemistry*, **71**, 132–141.

Jensen, K., Osmani, S.A., Hamann, T., Naur, P. and Møller, B.L. (2011) Homology modeling of the three membrane proteins of the dhurrin metabolon: catalytic sites, membrane surface association and protein–protein interactions. *Phytochemistry*, doi:10.1016/j.phytochem.2011.05.001.

Jones, D.A. (1998) Why are so many food plants cyanogenic? *Phytochemistry*, **47**, 155–162.

Jones, P.R., Møller, B.L. and Hoj, P.B. (1999) The UDP-glucose: p-hydroxymandelonitrile-o-glucosyltransferase that catalyzes the last step in synthesis of the cyanogenic glucoside dhurrin in *Sorghum bicolor*. Isolation, cloning, heterologous expression, and substrate specificity. *J. Biol. Chem.* **274**, 35483–35491.

Jørgensen, K., Bak, S., Busk, P.K., Sorensen, C., Olsen, C.E., Puonti-Kaerlas, J. and Møller, B.L. (2005a) Cassava plants with a depleted cyanogenic glucoside content in leaves and tubers. Distribution of cyanogenic glucosides, their site of synthesis and transport, and blockage of the biosynthesis by RNA interference technology. *Plant Physiol.* **139**, 363–374.

Jørgensen, K., Rasmussen, A.V., Morant, M., Nielsen, A.H., Bjarnholt, N., Zagrobelny, M., Bak, S. and Møller, B.L. (2005b) Metabolon formation and metabolic channeling in the biosynthesis of plant natural products. *Curr. Opin. Plant Biol.* **8**, 280–291.

Jørgensen, K., Morant, A.V., Morant, M., Jensen, N.B., Olsen, C.E., Kannangara, R., Motawia, M.S., Møller, B.L. and Bak, S. (2011) Biosynthesis of the cyanogenic glucosides linamarin and lotaustralin in cassava: isolation, biochemical characterization, and expression pattern of CYP71E7, the oxime-metabolizing cytochrome P450 enzyme. *Plant Physiol.* **155**, 282–292.

Kahn, R.A., Bak, S., Svendsen, I., Halkier, B.A. and Møller, B.L. (1997) Isolation and reconstitution of cytochrome P450ox and *in vitro* reconstitution of the entire biosynthetic pathway of the cyanogenic glucoside dhurrin from sorghum. *Plant Physiol.* **115**, 1661–1670.

Kakes, P. (1989) An analysis of the costs and benefits of the cyanogenic system in *Trifolium repens* L. *Theor. Appl. Genet.* **77**, 111–118.

Koch, B.M., Sibbesen, O., Halkier, B.A., Svendsen, I. and Møller, B.L. (1995) The primary sequence of cytochrome P450tyr, the multifunctional *N*-hydroxylase catalyzing the conversion of L-tyrosine to *p*-hydroxyphenylacetaldehyde oxime in the biosynthesis of the cyanogenic glucoside dhurrin in *Sorghum bicolor* (L.) Moench. *Arch. Biochem. Biophys.* **323**, 177–186.

Kongsawadworakul, P., Viboonjun, U., Romruensukharom, P., Chantuma, P., Ruderman, S. and Chrestin, H. (2009) The leaf, inner bark and latex cyanide potential of *Hevea brasiliensis*: evidence for involvement of cyanogenic glucosides in rubber yield. *Phytochemistry*, **70**, 730–739.

Kristensen, C., Morant, M., Olsen, C.E., Ekstrøm, C.T., Galbraith, D.W., Møller, B.L. and Bak, S. (2005) Metabolic engineering of dhurrin in transgenic *Arabidopsis* plants with marginal inadvertent effects on the metabolome and transcriptome. *Proc. Natl Acad. Sci. USA*, **102**, 1779–1784.

Lababidi, S., Mejlhede, N., Rasmussen, S.K., Backes, G., Al-Said, W., Baum, M. and Jahoor, A. (2009) Identification of barley mutants in the cultivar Lux at the Dhn loci through TILLING. *Plant Breed.* **128**, 332–336.

Lee, T.T. and Skoog, F. (1965) Effects of substituted phenols on bud formation and growth of tobacco tissue cultures. *Physiol. Plant.* **18**, 386–402.

Lieberei, R. (2007) South American leaf blight of the rubber tree (*Hevea* spp.): new steps in plant domestication using physiological features and molecular markers. *Ann. Bot.* **100**, 1125–1142.

Loyd, R.C. and Gray, E. (1970) Amount and distribution of hydrocyanic acid potential during the life cycle of plants of three sorghum cultivars. *Agron. J.* **62**, 394–397.

Miller, R., Jensen, R. and Woodrow, I. (2006) Frequency of cyanogenesis in tropical rainforests of Far North Queensland, Australia. *Ann. Bot.* **97**, 1017–1044.

Møller, B.L. (2010a) Dynamic metabolons. *Science*, **330**, 1328.

Møller, B.L. (2010b) Functional diversifications of cyanogenic glucosides. *Curr. Opin. Plant Biol.* **13**, 337–346.

Møller, B.L. and Conn, E.E. (1979) The biosynthesis of cyanogenic glucosides in higher plants. N-Hydroxytyrosine as an intermediate in the biosynthesis of dhurrin by *Sorghum bicolor* (L.) Moench. *J. Biol. Chem.* **254**, 8575–8583.

Møller, B.L. and Conn, E.E. (1980) The biosynthesis of cyanogenic glucosides in higher plants. Channeling of intermediates in dhurrin biosynthesis by a microsomal system from *Sorghum bicolor* (linn) Moench. *J. Biol. Chem.* **255**, 3049–3056.

Morant, A.V., Jørgensen, K., Jørgensen, B., Dam, W., Olsen, C.E., Møller, B.L. and Bak, S. (2007) Lessons learned from metabolic engineering of cyanogenic glucosides. *Metabolomics*, **3**, 383–398.

Morant, A.V., Jørgensen, K., Kristensen, C., Paquette, S.M., Sanchez-Perez, R., Moller, B.L. and Bak, S. (2008) β-Glucosidases as detonators of plant chemical defense. *Phytochemistry*, **69**, 1795–1813.

Nielsen, K.A., Tattersall, D.B., Jones, P.R. and Møller, B.L. (2008) Metabolon formation in dhurrin biosynthesis. *Phytochemistry*, **69**, 88–98.

Oleykowski, C.A., Bronson Mullins, C.R., Godwin, A.K. and Yeung, A.T. (1998) Mutation detection using a novel plant endonuclease. *Nucleic Acids Res.* **26**, 4597–4602.

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Olsen, K.M., Sutherland, B.L. and Small, L.L. (2007) Molecular evolution of the Li/li chemical defence polymorphism in white clover (*Trifolium repens* L.). *Mol. Ecol.* **16**, 4180–4193.

Olsen, K.M., Hsu, S.-C. and Small, L.L. (2008) Evidence on the molecular basis of the Ac/ac adaptive cyanogenesis polymorphism in White Clover (*Trifolium repens* L.). *Genetics*, **179**, 517–526.

Paquette, S.M., Jensen, K. and Bak, S. (2009) A web-based resource for the *Arabidopsis* P450, cytochromes b5, NADPH-cytochrome P450 reductases, and family 1 glycosyltransferases (http://www.P450.kvl.dk). *Phytochemistry*, **70**, 1940–1947.

Selmar, D., Lieberei, R. and Biehl, B. (1988) Mobilization and utilization of cyanogenic glycosides: the linustatin pathway. *Plant Physiol.* **86**, 711–716.

Seo, S., Mitsuhara, I., Feng, J., Iwai, T., Hasegawa, M. and Ohashi, Y. (2011) Cyanide, a coproduct of plant hormone ethylene biosynthesis, contributes to the resistance of rice to blast fungus. *Plant Physiol.* **155**, 502–514.

Sibbesen, O., Koch, B., Halkier, B.A. and Møller, B.L. (1994) Isolation of the heme-thiolate enzyme cytochrome $P\text{-}450_{TYR}$, which catalyzes the committed step in the biosynthesis of the cyanogenic glucoside dhurrin in *Sorghum bicolor* (L.) Moench. *Proc. Natl Acad. Sci. USA*, **91**, 9740–9744.

Takos, A., Lai, D., Mikkelsen, L., Abou Hachem, M., Shelton, D., Motawia, M.S., Olsen, C.E., Wang, T.L., Martin, C. and Rook, F. (2010) Genetic screening identifies cyanogenesis-deficient mutants of *Lotus japonicus* and reveals enzymatic specificity in hydroxynitrile glucoside metabolism. *Plant Cell*, **22**, 1605–1619.

Talame, V., Bovina, R., Sanguineti, M.C., Tuberosa, R., Lundqvist, U. and Salvi, S. (2008) TILLMore, a resource for the discovery of chemically induced mutants in barley. *Plant Biotechnol. J.* **6**, 477–485.

Tattersall, D.B., Bak, S., Jones, P.R., Olsen, C.E., Nielsen, J.K., Hansen, M.L., Høj, P.B. and Møller, B.L. (2001) Resistance to an herbivore through engineered cyanogenic glucoside synthesis. *Science*, **293**, 1826–1828.

Till, B.J., Burtner, C., Comai, L. and Henikoff, S. (2004) Mismatch cleavage by single-strand specific nucleases. *Nucleic Acids Res.* **32**, 2632–2641.

Till, B.J., Cooper, J., Tai, T.H., Colowit, P., Greene, E.A., Henikoff, S. and Comai, L. (2007) Discovery of chemically induced mutations in rice by TILLING. *BMC Plant Biol.* **7**, 19.

Wheeler, J. and Mulcahy, C. (1989) Consequences for animal production of cyanogenesis in sorghum forage and hay – a review. *Trop. Grassl.* **23**, 193–202.

Wheeler, J.L., Mulcahy, A.C., Walcott, J.J. and Rapp, G.G. (1990) Factors affecting the hydrogen cyanide potential of forage sorghum. *Aust. J. Agric. Res.* **41**, 1093–1100.

Xin, Z., Wang, M.L., Barkley, N.A., Burow, G., Franks, C., Pederson, G. and Burke, J. (2008) Applying genotyping (TILLING) and phenotyping analyses to elucidate gene function in a chemically induced sorghum mutant population. *BMC Plant Biol.* **8**, 103.

Zagrobelny, M., Bak, S., Olsen, C.E. and Møller, B.L. (2007) Intimate roles for cyanogenic glucosides in the life cycle of *Zygaena filipendulae* (Lepidoptera, Zygaenidae). *Insect Biochem. Mol. Biol.* **37**, 1189–1197.

Zerr, T. and Henikoff, S. (2005) Automated band mapping in electrophoretic gel images using background information. *Nucleic Acids Res.* **33**, 2806–2812.

Supporting information

Additional Supporting information may be found in the online version of this article:

Figure S1 Quantifiable HCNp Assay of the 264 selected M2 plants.

Figure S2 Sequence chromatograms of three 2-908-1 M4 siblings showing a homozygous wild-type line, a heterozygous line and a homozygous P414L line.

Table S1 Sequence of the primers and thermocycling conditions used for the PCR in the TILLING analysis.

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