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(54) Title: NOVEL STOCK FEED CROP PLANT

(57) Abstract: The present invention relates to a novel stock feed crop plant, particularly a sorghum crop plant with a reduced capacity to produce hydrogen cyanide. The plant comprises a mutation in one or more genes encoding the enzymes of the biosynthetic pathway for production of cyanogenic dhurrin, such that the plant either can not produce dhurrin or produces or accumulates dhurrin in low amounts relative to a wild-type plant.



## NOVEL STOCK FEED CROP PLANT

### FIELD OF THE INVENTION

- 5 The present invention relates to a novel stock feed crop plant, particularly a sorghum crop plant with a reduced capacity to produce hydrogen cyanide.

### INCORPORATION BY REFERENCE

This patent application claims priority from:

- 10 - United States Provisional Patent Application No 61/466358 filed 22 March 2011, and  
- Australian Provisional Patent Application No 2011901433 filed 15 April 2011.

The entire content of these applications is hereby incorporated by reference.

The following publications are referred to herein:

- 15 - International Patent Publication No WO 95/16041 titled "Cytochrome P-450 monooxygenases",  
- Bak S *et al.* (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  
20 cyanogenic glucoside dhurrin. *Plant Molecular Biology* 36:393-405, and  
- Thorsøe KS *et al.* (2005). Determination of catalytic key amino acids and UDP sugar donor specificity of the cyanohydrin glycosyltransferase UGT85B1 from *Sorghum bicolor*. Molecular modeling substantiated by site-specific mutagenesis and biochemical analyses. *Plant Physiol* 139:664-673.

- 25 The entire content of these publications is also hereby incorporated by reference.

### BACKGROUND OF THE INVENTION

- Sorghum bicolor* is an important grain and forage (stock feed) crop plant in many parts of the world and, particularly, semi-dry tropical areas as it is highly drought tolerant. However, sorghum is one  
30 of many plant species that produce cyanogenic glycosides (Jones, 1998); tyrosine-derived cyanogenic glucoside dhurrin (Akazawa *et al.*, 1960) in the case of this species, which can be a major risk to livestock. That is, upon tissue disruption of the sorghum leaf (such as caused by cattle feeding), dhurrin is hydrolysed by  $\beta$ -glucosidases resulting in the release of hydrogen cyanide (HCN; Morant *et al.*, 2008) in a process known as cyanogenesis and which serves both as a plant  
35 defence mechanism primarily towards herbivores (Gleadow and Woodrow, 2002a) and as a means

for storage of nitrogen and important antioxidants (Møller, 2010). Unfortunately, under abiotic and biotic stress such as drought or following application of high nitrogen, the concentration of dhurrin can consequently increase to levels which can lead to livestock death because of the HCN release following ingestion (Wheeler and Mulcahy, 1989). In addition, very young plants are also toxic.

- 5 Consequently, as a result of farmer concerns over possible cyanide toxicity, the price of forage sorghum is often regarded as being depressed. Indeed, in Australia alone, it has been estimated that such farmer concerns decreases the value of sorghum as stock feed by approximately \$20 million on an annual basis.
- 10 The biosynthesis and catabolism of dhurrin and the resulting liberation of hydrogen cyanide have been the subject of extensive studies in sorghum (see, for example, Sibbesen *et al.*, 1994, Koch *et al.*, 1995, Bak *et al.*, 1998, and Jones *et al.*, 1999). Dhurrin is produced via a biosynthetic pathway organised within a metabolon involving three key genes encoding two cytochrome P450s (CYP79A1 and CYP71E1) and a UDP-glucosyltransferase (UGT85B1) (Figure 1). The
- 15 intermediates in the pathway are labile and toxic and are channelled into dhurrin formation by the metabolon to prevent undesired metabolic cross-talk (Jørgensen *et al.*, 2005, and Nielsen *et al.*, 2008). Sorghum is the first plant in which all three genes involved in cyanogenic glucoside synthesis have been identified. The present applicants have now utilised this knowledge to design a reverse genetic screen based on TILLING (Targeted Induced Local Lesions in Genomes) to
- 20 generate and identify mutations that result in alterations in the dhurrin biosynthetic pathway.

- TILLING has been successfully used to identify particular genes of interest in several plant species including crop plants such as maize, rice and barley (Till *et al.*, 2007, Talame *et al.*, 2008, and Lababidi *et al.*, 2009). TILLING relies on the enzymatic cleavage of single nucleotide mismatches
- 25 in DNA heteroduplexes by the endonuclease CEL1 to identify mutations caused by exposure to the chemical, ethyl methanesulfonate (EMS). Xin *et al.*, 2008 has previously documented the feasibility of TILLING in sorghum by screening a small EMS-mutagenised population of 768 sorghum plants for alterations in four selected genes of agronomic value. Further, cyanogenesis deficient (*cyd*) mutants lacking in biosynthesis as well as degradation of cyanogenic glucosides
- 30 have been identified in *Lotus japonicus* using TILLING; of which breakdown mutants with identified mutations in  $\beta$ -glucosidase genes have been characterised in detail (Takos *et al.*, 2010). However, similar sorghum mutants (ie targeting the  $\beta$ -glucosidase genes and disrupting this enzyme function) would not be suitable for the production of acyanogenic sorghum stock feed crop

plants, because ruminants such as cattle carry microorganisms in the rumen that have  $\beta$ -glucosidase activity and would be able to breakdown any dhurrin ingested by the animal regardless of the presence or absence of plant  $\beta$ -glucosidase activity (Wheeler and Mulcahy, 1989). Accordingly, the aim of the present applicants was to attempt to generate and select mutant plants on the basis of their capacity to produce HCN (HCN potential, HCNp), rather than cyanogenesis, which is dependent on the presence of specific endogenous  $\beta$ -glucosidases.

#### SUMMARY OF THE INVENTION

In a first aspect, the present invention provides a plant cell of, or derived from, a plant species that, in wild-type, produces cyanogenic dhurrin, wherein the plant cell comprises a mutation in one or more genes encoding the enzymes of the biosynthetic pathway for production of said dhurrin in said plant cell, such that said plant cell either can not produce dhurrin or produces or accumulates dhurrin in low amounts relative to a corresponding plant cell of said wild-type.

The plant cell may form part of a multicellular structure such as a whole plant, plant tissue, a component or organ of a plant, and reproductive material thereof (eg viable seed).

The mutation may be in the gene of one or more of the following enzymes; cytochrome P450 CYP79A1, cytochrome P450 CYP71E1 and UDP-glucosyltransferase UGT85B1.

In a second aspect, the present invention provides a mutant polynucleotide molecule comprising;

- (i) a nucleotide sequence comprising a defective regulatory region of a gene encoding cytochrome P450 CYP79A1, cytochrome P450 CYP71E1 or UDP-glucosyltransferase UGT85B1, and/or
- (ii) a nucleotide sequence encoding a truncated or otherwise mutated cytochrome P450 CYP79A1, cytochrome P450 CYP71E1 or UDP-glucosyltransferase UGT85B1, wherein said polynucleotide molecule is in a substantially isolated form.

In a third aspect, the present invention provides a vector (eg a plasmid or expression cassette) including a mutant polynucleotide molecule comprising;

- (i) a nucleotide sequence comprising a defective regulatory region of a gene encoding cytochrome P450 CYP79A1, cytochrome P450 CYP71E1 or UDP-glucosyltransferase UGT85B1, and/or

(ii) a nucleotide sequence encoding a truncated or otherwise mutated cytochrome P450 CYP79A1, cytochrome P450 CYP71E1 or UDP-glucosyltransferase UGT85B1.

#### BRIEF DESCRIPTION OF THE FIGURES

5 **Figure 1** provides a schematic representation of the biosynthetic pathway of cyanogenesis in sorghum;

**Figure 2** provides: (A) a schematic representation of the sorghum CYP79A1 gene showing the relative locations of EMS-induced mutations in 11 mutant plant lines; (B) a structural homology  
10 model of the CYP79A1 enzyme showing the location of amino acid residues Met<sup>98</sup>, Glu<sup>145</sup>, Ala<sup>154</sup>, Pro<sup>414</sup> and Glu<sup>529</sup> that are substituted in the lines 5-150-1 (M98I), 5-84-1 (E145K), 5-132-1 (A154T), 2-908-1 (P414L) and 4-327-2 (E529K) respectively; and (C) a ribbon diagram of the substrate-binding site of the CYP79A1 enzyme with the substrate L-tyrosine present and showing its relationship to the mutation P414L;

15 **Figure 3** provides an image of Northern blot analysis of CYP79A1 mRNA in 34 day old M4 mutant lines derived from the M2 mutant plant line 2-908-1 (containing a P414L mutation in CYP79A1) as compared to mature (34 day old) and young (6 day old seedlings) of a wild-type breeding line of sorghum (ie the line used to generate the EMS-mutated population);

20 **Figure 4** provides images of the phenotype in seedlings of eight mutant plant lines: (A) 2-908-1-1-5, (B) 2-908-1-5-2, (C) 4-565-1-11-2, (D) 2-1307-2-10-1, (E) 2-1307-2-10-5, (F) 4-970-1-1-2, (G) 4-970-1-6-1, (H) 4-970-1-10-3, and (I) non-mutated (control) parent line. The plants shown in (A) and (B) contained a P414L mutation in CYP79A1, while those shown in (C) to (H) are believed to  
25 be regulatory mutants or cyanogenic glucoside mutants. The plant lines 2-908-1-1-5 and 2-908-1-5-2 showed a markedly shorter seedling phenotype in plants less than 1 week old, but as the plants matured there was little size difference compared to normal, non-mutated plants (I). The measuring stick shown is 1 metre in height, marked in 10 cm increments;

30 **Figure 5** provides images of mutant plants growing in either the field or greenhouse in Toowoomba, QLD, Australia: (A) An M3 population growing in the field illustrating the good growth and normal phenotype of several mutant lines all containing a P414L mutation and showing low HCNp; (B) An M4 population growing in a greenhouse at 34 days post-planting; (C) Non-

mutated control lines growing in a greenhouse; (D) Mutant line 2-908-1 in the right row and control non-mutated plants in the left row, showing no phenotypic effects of the mutation; and

**Figure 6** provides graphical results showing the HCNp (as measured by quantitative laboratory assay; Gleadow *et al.*, 2011) of 2-908-1 through generation M2 to M4 compared to HCNp of non-mutated control plants. The y axis shows HCNp in mg/g dry weight. The control sample, containing 0.39 mg HCNp/g dry weight, represents the average HCNp from 20 individual non-mutated individual plants of the parental breeding variety of sorghum which were grown and harvested at the same time as the M4 plants. Lines 2-908-1-1, 2-908-1-4 and 2-908-1-5 represent individual M3 plants that were deemed homozygous for the P414L mutation. This is clearly evident in that all M4 offspring from these plants, following self fertilisation, had low levels (25% or less) than the average of wild-type control plants at the same stage of growth (represented by M4 individuals 2-908-1-1-1 through 2-908-1-1-7; 2-908-1-2-1 through 2-908-1-2-4; and 2-908-1-5-1 through 2-908-1-5-5).

#### DETAILED DESCRIPTION OF THE INVENTION

The present applicants sought to generate and select mutants of dhurrin-producing plant species, particularly sorghum, with reduced capacity to produce HCN (HCN potential, HCNp). Such mutants are suitable for use as novel stock feed crop plants.

Thus, in a first aspect, the present invention provides a plant cell of, or derived from, a plant species that, in wild-type, produces cyanogenic dhurrin, wherein the plant cell comprises a mutation in one or more genes encoding the enzymes of the biosynthetic pathway for production of said dhurrin in said plant cell, such that said plant cell either can not produce dhurrin or produces or accumulates dhurrin in low amounts relative to a corresponding plant cell of said wild-type.

The plant cell may be selected from, for example, species within the *Sorghum* genus including, but not limited to *Sorghum bicolor* L. (cultivated sorghum), *Sorghum bicolor* subspecies *drummondii* (Sudan grass – also known as *Sorghum sudanense*) and *Sorghum halepense* (Johnson grass).

Otherwise, the plant cell may be, for example, a hybrid derived from, for example, a sorghum species (eg *Sorghum X alnum* and *Sorghum X drummondii* hybrids).

Preferably, the plant cell is of the species *S. bicolor* or is a hybrid thereof.

Preferably, the plant cell forms part of a multicellular structure, particularly a whole plant (including, for example, seedlings and mature plants), but including plant tissue (including a plant tissue culture), a component or organ of a plant, and reproductive material thereof (eg viable seed).

- 5 The mutation may have been generated using any of the methods for mutagenesis of plants well known to persons skilled in the art including treatment with a chemical mutagenic agent such as EMS and sodium azide or mutagenic radiation (eg X-, UV- or  $\gamma$ -irradiation). Alternatively, the mutation may be introduced by, for example, any of the suitable techniques of genetic manipulation well known to persons skilled in the art including site-directed mutagenesis. However, by virtue of  
10 continued poor public perception and market resistance to GMOs, generation of the mutation of the present invention by such techniques of genetic manipulation is preferably avoided.

The mutation may be in the gene of one or more of the following enzymes; cytochrome P450 CYP79A1, cytochrome P450 CYP71E1 and UDP-glucosyltransferase UGT85B1.

15

- The mutation may comprise a point, insertion and/or deletion mutation located within a coding or non-coding (eg regulatory) gene region, which renders the gene defective inasmuch as the gene is either no longer able to be expressed in the plant cell or otherwise encodes an enzyme with no or diminished activity. As such, the mutation may, for example, code for an amino acid substitution,  
20 insertion and/or deletion in the enzyme. Alternatively, or additionally, the mutation may code for a stop codon resulting in the expression of a truncated enzyme.

- Preferably, the mutation is in the gene encoding CYP79A1. More preferably, the mutation is in a coding region of the CYP79A1 gene, and particularly such a region which codes for one or more  
25 amino acids comprising the substrate (ie L-tyrosine) binding site of the enzyme or which might otherwise perturb the substrate cavity and/or heme binding ability of the enzyme such that there is no or diminished catalytic activity. Examples of suitable mutations in the CYP79A1 gene code for one or more of the following amino acid substitutions; E145K and P414L (nb. the standard single letter amino acid code is used throughout the specification).

30

The polynucleotide sequence for sorghum CYP79A1 has been published by Møller *et al.* (eg see SEQ ID NO: 1 of International Patent Specification No WO 95/16041; the entire content of which is herein incorporated by reference). The E145K mutation may comprise a G→A nucleotide transition at position 433 in the coding sequence of the gene, while the P414L mutation may

comprise a C→T nucleotide transition at position 1240 in the coding sequence of the gene. The P414L mutation is contained in homozygote form in certain offspring of M2 mutant line 2-908-1; namely mutant M3 plant lines 2-908-1-1, 2-908-1-2 and 2-908-1-5 and their direct offspring, particularly 2-908-1-5-4 as described hereinafter and deposited with the National Collections of Industrial, Food and Marine Bacteria (NCIMB) under the *Budapest Treaty* on 14 April 2011, Accession No 41826 (NCIMB 41826).

Preferably, the plant cell of the present invention is homozygous for the mutation.

10 The plant cell of the present invention either can not produce dhurrin or produces or accumulates dhurrin in low amounts relative to a corresponding plant cell of said wild-type grown under equivalent conditions. Such low amounts include amounts (as measured by the quantified HCNp assay of Gleadow *et al.*, 2011 that are < 50%, more preferably  $\leq 25\%$ , of that produced by a corresponding plant cell (eg a plant cell of an equivalent plant tissue, component (preferably leaf) or organ) of said wild-type grown under equivalent conditions.

It is to be understood that the term "wild-type" as used herein refers to a dhurrin-producing species that possesses the common, typically dominant, form of the genes encoding the enzymes of the biosynthetic pathway for production of dhurrin in said species, found in natural (ie wild) populations. For the purposes of the present invention, a near isogenic inbred parent line of *S. bicolor* (L.) Moench represents an example of a wild-type sorghum.

In one preferred embodiment, the invention of the first aspect provides a sorghum plant or hybrid which comprises a mutation in a gene encoding CYP79A1 which renders the gene defective in that it is not expressed or otherwise encodes a CYP79A1 enzyme with no or diminished activity, such that said plant either can not produce dhurrin or produces or accumulates dhurrin in low amounts relative to a corresponding plant cell of a wild-type sorghum species.

Preferably, the CYP79A1 gene mutation of said sorghum plant or hybrid codes for one or more of the following amino acid substitutions; E145K and P414L. More preferably, the CYP79A1 gene mutation codes for a P414L substitution and, accordingly, the sorghum plant or hybrid of the preferred embodiment may comprise the mutated CYP79A1 gene of the mutant plant line 2-908-1-5-4 (NCIMB Accession No 41826).

In another preferred embodiment, the invention of the first aspect provides a plant of mutant plant line 2-908-1-5-4 (NCIMB Accession No 41826).

The mutated CYP79A1 gene of the mutant plant line 2-908-1-5-4 (NCIMB Accession No 41826) is stable and homozygous. Consequently, it is possible to transfer the mutation to different plants, particularly different sorghum plants, through cross-breeding (hybridisation) and appropriate selection. Thus, it is to be understood that the present invention extends to, particularly, daughter plants (including hybrids), granddaughter plants, great granddaughter plants etc of a plant of the mutant plant line 2-908-1-5-4 (NCIMB Accession No 41826).

In a further preferred embodiment, the invention of the first aspect provides a plant (or a plant cell) of, or derived from, a plant species that, in wild-type, produces cyanogenic dhurrin, wherein the plant (or plant cell) comprises a mutation in one or more genes encoding the enzymes of the biosynthetic pathway for production of said dhurrin, which thereby provides the plant (or plant cell) with an acyanogenic or low HCNp trait as shown by mutant plant line 2-908-1-5-4, wherein representative seeds of which have been deposited with the NCIMB under Accession No 41826.

In a second aspect, the present invention provides a mutant polynucleotide molecule comprising;

- (i) a nucleotide sequence comprising a defective regulatory region of a gene encoding cytochrome P450 CYP79A1, cytochrome P450 CYP71E1 or UDP-glucosyltransferase UGT85B1, and/or
- (ii) a nucleotide sequence encoding a truncated or otherwise mutated cytochrome P450 CYP79A1, cytochrome P450 CYP71E1 or UDP-glucosyltransferase UGT85B1, wherein said polynucleotide molecule is in a substantially isolated form.

In a third aspect, the present invention provides a vector (eg a plasmid or expression cassette) including a mutant polynucleotide molecule comprising;

- (i) a nucleotide sequence comprising a defective regulatory region of a gene encoding cytochrome P450 CYP79A1, cytochrome P450 CYP71E1 or UDP-glucosyltransferase UGT85B1, and/or
- (ii) a nucleotide sequence encoding a truncated or otherwise mutated cytochrome P450 CYP79A1, cytochrome P450 CYP71E1 or UDP-glucosyltransferase UGT85B1.

As mentioned above, the polynucleotide sequence for sorghum CYP79A1 has been published by Møller *et al.* (International Patent Specification No WO 95/16041). The polynucleotide sequences for sorghum CYP71E1 and UGT85B1 have been published by Bak *et al.*, 1998 and Thorsøe *et al.*, 2005, respectively; the entire content of which are herein incorporated by reference). Mutant forms of these polynucleotide molecules may comprise a point, insertion and/or deletion mutation. The mutation may be located within a non-coding (eg regulatory) region of the polynucleotide molecule, such that the region is defective inasmuch as it is no longer able to control or promote expression of any operably linked coding region. However, preferably, the mutation is located within a coding region of the polynucleotide molecule and encodes an enzyme with no or diminished activity. As such, a coding region mutation may, for example, code for an amino acid substitution, insertion and/or deletion in the enzyme. Alternatively, or additionally, the mutation may code for a stop codon such that expression of the polynucleotide molecule results in a truncated enzyme.

Preferably, the mutation is located within a region of a CYP79A1 polynucleotide molecule which codes for one or more amino acids comprising the substrate (ie L-tyrosine) binding site of the enzyme or which might otherwise perturb the substrate cavity and/or heme binding ability of the enzyme such that there is no or diminished catalytic activity (eg mutations in the sorghum CYP79A1 polynucleotide molecule that code for one or more of the following amino acid substitutions; E145K and P414L).

In a preferred embodiment, the polynucleotide molecule of the invention of the second or third aspect comprises a nucleotide sequence substantially corresponding to that of the mutated CYP79A1 gene of the mutant plant line 2-908-1-5-4 (NCIMB Accession No 41826).

It is to be understood that the term "substantially corresponding" as used herein in relation to nucleotide sequences is intended to encompass minor variations in the nucleotide sequences which, due to degeneracy in the DNA code, do not result in any significant change in the encoded CYP79A1 enzyme.

The polynucleotide molecule and vector of the second and third aspects of the invention respectively, may be introduced into a plant cell by, for example, any of the suitable methods for transformation well known to the person skilled in the art (eg *Agrobacterium*-mediated transformation (see, in particular, the method of *Agrobacterium*-mediated sorghum transformation

- described by Zhao *et al.*, 2000), microprojectile-based transformation and DNA uptake processes involving, for example, electroporation). Through homologous recombination, the polynucleotide molecule may stably replace one or both copies of homologous sequences on the host plant cell chromosomes, and thereby confer a change in HCNp (ie effect a reduction in the capacity of the plant cell to produce HCN). The host plant cell may be of a dhurrin-producing species or, otherwise, may be of a species which produces another cyanogenic glucoside compound and possesses a sufficiently homologous sequence within the genome (eg the homologous sequences encoding corresponding enzymes in cassava, rape (canola) and nasturtium; WO 95/16041).
- 10 In a further aspect, the present invention provides a product of a plant according to the invention such as, for example, a harvested product such as grain or straw, or a processed product such as syrup (eg sorghum molasses) or fibre.

- 15 In order that the nature of the present invention may be more clearly understood, preferred forms thereof will now be described with reference to the following non-limiting examples.

## EXAMPLES

### Example 1

20

#### *Materials and Methods*

##### Plant material

- A near-isogenic, *Sorghum bicolor* (L.) Moench line (Pacific Seeds Pty Ltd, Toowoomba, QLD, Australia) was treated with 0.15-0.4% ethyl methanesulfonate (EMS; Sigma-Aldrich Corporation, St Louis, MO, United States of America) for 16 hours with shaking (~50 rpm) and washed thoroughly under running water. A total of 53000 seeds (in batches of ~1000 seed / 500 mL) were EMS treated. All germinating plants were grown under normal field conditions (10 cm intervals between plants, 75 cm intervals between rows) in the field. To ensure self-pollination, each head was bagged prior to anthesis. Bags were dated, harvested manually and threshed individually, resulting in nearly 4200 individual M2 mutant lines. Germination rates and seed set varied depending on the severity of the EMS treatment.
- 25  
30

Where available, up to five seeds from each M2 line were then sown in the field at the start of spring in 2008. The plants were screened for hydrogen cyanide levels (using the assays described

below) in late spring when the plants were ~6 weeks old. The main panicle of all M2 plants was bagged to prevent cross-pollination and the seed was collected and threshed individually.

Selected M3 mutant lines were sown in spring of 2009 in the field, as described above, whilst lines of the M4 generation were planted in the greenhouse in autumn of 2010. Again, the main panicle for all plants of the M3 and M4 generations were bagged to allow self-fertilisation and the generation of homozygous mutants. All seed was collected and stored for analysis.

#### Assays for the cyanide content of mutant plant lines

- 10 Feigl-Anger (FA) papers were prepared by dipping filter paper (Whatman No. 541; Whatman plc, Maidstone, Kent, United Kingdom) into a solution containing equal volumes of copper ethyl acetate and tetrabase (4-4'-methylene-bis-N,N-dimethyl-aniline; Sigma 40H2635, Sigma-Aldrich) and air drying (Feigl and Anger, 1966, as modified by Gleadow *et al.*, 2011). The FA papers were used to screen all 4200 plants in the M2 generation to identify mutants with low or limited capacity
- 15 for cyanogenesis (Gleadow *et al.*, 2011; Takos *et al.*, 2010). Three discs (diameter ~0.5cm) were taken from the youngest fully unfurled leaf of each plant and placed in a 96-well plate and covered with a single sheet of FA paper and the lid closed and secured firmly with 4 butterfly clips. The sealed plate was then placed on dry-ice until completely frozen (about 1 hour). The plates were then thawed at ambient temperature thus lysing the cells, and allowing any dhurrin and dhurrinase
- 20 present in the tissue to mix. Plates were and incubated at ~25°C for 2 hours, allowing HCN to evolve. When HCN is present, it reacts with the FA paper causing a blue colour to develop above each well. The intensity of the colour is proportional to the amount of HCN present (Miller *et al.*, 2006). Following the freeze-thaw cycle, the FA papers were carefully removed and photographed.
- 25 Based on the screening using the FA papers, 264 individual M2 plants showing extreme differences (either low or high) in cyanide potential (HCNp) compared to control non-mutated parents, were selected for accurate determination of their HCNp using the method of Woodrow *et al.*, 2002 as modified by Gleadow *et al.*, 2011. The youngest fully unfurled leaf was collected and dried at 37°C for one week and then ground to fine powder in a mixer mill. Previous experiments had shown that
- 30 the dhurrin was not degraded in the process (Gleadow *et al.*, 2012). A sub-sample of the ground tissue (~0.01g) was accurately weighed out and placed in a vial; 500 µL of 0.1 M citrate buffer (pH 5.5) containing β-glucosidase emulsin from almond (*Prunus amygdalis*; β-D-glucoside glucohydrolase; EC 3.2.1.21) (1.12 units mL<sup>-1</sup>) was added and a PCR tube containing 200 µL of

1M NaOH was placed in the vial which was then tightly sealed. Vials were incubated for 15 hours at 37°C. Any cyanogenic glucoside in the leaf sample is released by the  $\beta$ -glucosidase and the resulting HCN is absorbed by the NaOH to give NaCN, which is then quantified using a colourimetric assay using NaCN in 0.1 M NaOH as the standard (Woodrow *et al.*, 2002).

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#### Genomic DNA extraction

The MagAttract DNA extraction kit (Qiagen Inc, Germantown, MD, United States of America) was used to isolate total genomic DNA from the 264 individual M2 plants selected for the TILLING (Targetting Induced Local Lesions in Genomes) screen. The concentration of the genomic DNA was determined using a nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, United States of America) and 10 $\mu$ L aliquots containing 100ng gDNA was pipetted into individual wells of a 96 well PCR plate. Including controls, this resulted in three plates of gDNA representing the 264 individual M2 plants. Multiple plates were prepared to enable the analysis by PCR and TILLING using primers specific to CYP79A1 and UGT85B1. For other experiments, genomic DNA was isolated using the FastDNA Spin Kit and the FastPrep-24 Instrument (MP Biomedicals, Irvine, CA, United States of America) and quantified by nanodrop spectrophotometer.

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#### PCR, TILLING and mutation detection

20 A nested PCR approach was used to amplify the target genes for TILLING. Two primer sets were designed, either to the 5' or to the 3' region of CYP79A1 and UGT85B1 (Table 1). Primers for CYP71E1 were not designed as it was considered that mutants in this gene only are likely to be non-viable. The sequences for CYP79A1 and UGT85B1 were obtained from Genbank, and Primer3 and PerlPrimer programs were used to design the PCR primer sets. All PCR reactions were performed in a 20  $\mu$ L reaction volume using the proof reading enzyme, *Pfu* DNA polymerase (Promega Corporation, Madison, WI, United States of America) with 1  $\times$  *Pfu* PCR buffer containing 2 mM MgCl<sub>2</sub>, 0.2 mM dNTPs, 0.5 U *Pfu* polymerase, 0.2  $\mu$ M gene-specific forward and reverse primers (GeneWorks Pty Ltd, Hindmarsh, SA, Australia). Following the first PCR reaction, the products were pooled three-fold in a matrix design to allow identification of the mutated individual, and re-amplified using a combination of gene specific nested primers with universal M13 sequences at the 5' end (termed tailed nested primers) and the corresponding forward and reverse M13 primers, which were fluorescently labelled with IR dye 700nm and 800, respectively (Eurofins MWG Operon, Ebersberg, Germany) to allow visualisation following

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gel separation on a Li-Cor DNA analyser ([http://www.licor.com/bio/products/imaging\\_systems/4300/4300\\_dna\\_analyzer.jsp](http://www.licor.com/bio/products/imaging_systems/4300/4300_dna_analyzer.jsp)). The reaction volume was again 20  $\mu$ L containing 1  $\times$  *Pfu* PCR buffer with 2 mM  $MgCl_2$ , 0.2 mM dNTPs, 0.5 U *Pfu* polymerase, 0.2  $\mu$ M gene nested specific forward and reverse primers (GeneWorks) and 1  $\mu$ M M13 labelled forward and reverse primers (primers are detailed in Table 1). The thermocycling conditions (Eppendorf Mastercycler, Hamburg, Germany) were as follows; PCR 1 95°C - 2 min; 95°C - 30 sec step down 62°C, -1°C / cycle for 11 cycles - 30 sec, 72°C - 2.5 min; 95°C - 30 sec, 51°C - 30 sec, 72°C - 2.5 min for 30 cycles; 72°C - 10 min 4°C hold; PCR 2 95°C - 30 sec, 58°C - 30 sec, 72°C - 2.5 min for 30 cycles; 72°C - 10 min 4°C hold.

Following the second PCR, the amplified products were denatured and re-annealed slowly (99°C - 10 min; 70°C - 20 sec, -0.3°C / cycle  $\times$  70 cycles; 48°C - 20 sec, -0.3°C / cycle  $\times$  50; 4°C hold) to form heteroduplex DNA. Mutations were detected by digestion of the heteroduplexed DNA using the CEL1 enzyme isolated from celery according to Oleykowski *et al.*, 1998. CEL1 cleaves at mismatched nucleotides and these smaller products are visualised by electrophoresis on the LiCor 6400. GelBuddy ([www.gelbuddy.org](http://www.gelbuddy.org); Zerr and Henikoff, 2005) was used to analyse the gels and identify the mutations in the individual M2 plants.

#### Sequencing of cyanide-deficient mutants

Following identification of putative mutants, genomic DNA from a master plate was used to PCR the product for sequencing. The PCR products were purified using the Promega Wizard Kit (Promega) with an added final ethanol precipitation step to optimise DNA purity. The concentration was determined using the nanodrop spectrophotometer and 40-60 ng used in the BigDye Terminator sequencing kit (Applied Biosystems Inc, Foster City, CA, United States of America).

#### Preparation of microsomes

Seventy seeds of each of *Sorghum bicolor* wild-type (near isogenic breeding line) and of selected TILLING lines were sown in fine grade vermiculite and germinated in the dark for 4 days at 23°C. Each class of seedlings was harvested separately, counted, weighed and ground in a small chilled mortar with 600 mg polyvinylpyrrolidone (PVP) wetted with 8 mL isolation buffer (250 mM sucrose, 100 mM Tricine (pH 7.9), 50 mM NaCl, 2 mM DTT). Homogenate was filtered through nylon cloth (22  $\mu$ m mesh) and centrifuged (10 min, 10000 g). Supernatant was centrifuged (1 h,

100000 g) and the microsomal pellet resuspended in 8 mL isolation buffer using a tiny marten paintbrush to wash away soluble substrates and co-factors bound to the microsomal membranes. Following recentrifugation as above, the microsomal pellet was resuspended in 120  $\mu$ L resuspension buffer (50 mM Tricine (pH 7.9), 20 mM NaCl, 2 mM DTT) and the final volume adjusted so that 2.33  $\mu$ L 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 in each of the microsomal preparations was measured by the ability to reduce cytochrome *c* (Horseheart cytochrome *c*; Sigma-Aldrich) as monitored spectrometrically at 550 nm. Sample cuvette (total volume: 1 mL) contained 50  $\mu$ L 1 mM cytochrome *c* and 5  $\mu$ L microsomal membrane preparation in 50 mM  $KP_i$  (pH 7.9). Following monitoring of the background reduction rate for 3 min, 10  $\mu$ L 61.6 mM NADPH was added and the initial rate of cytochrome *c* reduction was measured. No NADPH was added to the reference cuvette. At the assay conditions used, 1 nmol of NADPH-cytochrome P450 oxidoreductase will reduce about 3000 nmol of cytochrome *c* per min enabling quantification of the activity in each sorghum seedling (Guengerich *et al.*, 2009).

#### Analysis of CYP79A1 activity

The activity of CYP79A1 in the microsomal membrane preparations was assayed in reaction mixtures (total volume: 20.2  $\mu$ L) containing 2.5  $\mu$ L L-[UL- $^{14}C$ ]-tyrosine (482mCi/mmol), 2.5  $\mu$ L 8 mM NADPH and 5  $\mu$ L resuspension buffer. The reaction was initiated by addition of 7.2  $\mu$ L microsomes. At the end of 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 (vol/vol)), 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  $\mu$ L 1mM L-tyrosine to saturate the enzyme system with substrate over the entire incubation period. The precise position of the different intermediates on the TLC plates was determined by co-application of unlabelled reference compounds, the position of which was monitored by their UV-absorption.

#### Quantification of dhurrin content in dark grown seedlings

Ten seedlings were collected of wild-type and of each TILLING line, weighed and homogenised in 600  $\mu\text{L}$  85% MeOH in an Eppendorf tube using a pointed pestle. Following extraction (4 h, RT, gentle shaking), the homogenate was centrifuged (10 min, 3000 g). Aliquots (20  $\mu\text{L}$ ) were diluted with 60  $\mu\text{L}$   $\text{H}_2\text{O}$  and filtered through a 0.45  $\mu\text{M}$  membrane by centrifugation. Dhurrin content was determined by injection of 0.1 and 2  $\mu\text{L}$  aliquots in a liquid chromatography mass spectrometer (LC-MS) and analysed as outlined below.

#### 10 LC-MS experimental data

Analytical LC-MS was carried out using an Agilent 1100 Series LC (Agilent Technologies Inc, Santa Clara, CA, United States of America) hyphenated to a Bruker HCT-Ultra ion trap mass spectrometer (Bruker Daltonics GmbH, Bremen, Germany). A Zorbax SB-C18 column (Agilent Technologies; 1.8  $\mu\text{M}$ ,  $2.1 \times 50\text{mm}$ ) was used at a flow rate of  $0.2 \text{ mL min}^{-1}$ , and the oven temperature was maintained at  $35^\circ$ . The mobile phases were: A, water with 0.1% (v/v)  $\text{HCOOH}$  and 50  $\mu\text{M}$   $\text{NaCl}$ ; B, acetonitrile with 0.1% (v/v)  $\text{HCOOH}$ . The gradient program was: 0 to 0.5 min, isocratic 2% B; 0.5 to 7.5 min, linear gradient 2 to 40% B; 7.5 to 8.5 min, linear gradient 40% to 90% B; 8.5 to 11.5 min isocratic 90% B; 11.60 to 17 min, isocratic 2% B. The flow rate was increased to  $0.3 \text{ mL min}^{-1}$  in the interval 11.2 to 13.5 min. The LC MS was run in positive electrospray mode.

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#### Immunoblotting

Antibodies were raised against synthetic peptides of CYP79A1 and CYP71E1. Equal volumes of microsomal sample preparations from the non-mutated parent and mutant lines were aliquoted into vials for immunoblotting against these antibodies. The proteins from the microsomal preps were separated on 12% gels from Criterion (BioRad Laboratories Inc, Berkeley, CA, United States of America) in MOPS buffer for 1 hour at 200V. The proteins were then transferred to Nitrocellulose membranes for 45 min at 100V. The membranes were blocked for 1 hour at room temperature in 5% skim milk powder solution (PBS-T), washed and the immunoreactions were carried out in 5% skim milk powder solution (PBS-T) with antibodies CYP79A1 at 1:2000 dilution and CYP71E1 at 1:5000 dilution for 1 hour at room temperature. The blot was incubated in the secondary antibody for 1 hour at room temperature at a dilution of 1:2000.

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Table 1

| Gene                    | Primer sequence (5' to 3')  | PCR Product Length (bp) |
|-------------------------|-----------------------------|-------------------------|
| CYP79A1<br>5'<br>region | Forward                     |                         |
|                         | Reverse                     | 1070                    |
|                         | nested & M13 tailed Forward | (SEQ ID NO: 3)          |
|                         | nested & M13 tailed Reverse | (SEQ ID NO: 4)          |
| CYP79A1<br>3'<br>region | Forward                     |                         |
|                         | Reverse                     | 1069                    |
|                         | nested & M13 tailed Forward | (SEQ ID NO: 7)          |
|                         | nested & M13 tailed Reverse | (SEQ ID NO: 8)          |
| UGT85B1<br>5'<br>region | Forward                     |                         |
|                         | Reverse                     | 967                     |
|                         | nested & M13 tailed Forward | (SEQ ID NO: 11)         |
|                         | nested & M13 tailed Reverse | (SEQ ID NO: 12)         |
| UGT85B1<br>3'<br>region | Forward                     |                         |
|                         | Reverse                     | 839                     |
|                         | nested & M13 tailed Forward | (SEQ ID NO: 15)         |
|                         | nested & M13 tailed Reverse | (SEQ ID NO: 16)         |
| M13<br>primers          | Forward IRD700              |                         |
|                         | Reverse IRD800              |                         |

## Results

### Isolation of cyanogenesis mutants in *S. bicolor*

To generate a mutant population of *S. bicolor*, 53000 seed were treated with EMS (0.15-0.4%) resulting in the germination and growth of ~16300 M1 plants. The higher concentrations of EMS affected the fertility of a large proportion of the plants and only about 4200 plants set seed, with some panicles producing <5 seeds. Due to seed availability and space constraints up to five M2 seed were planted per line, resulting in a population of ~6000 M2 plants. FA paper screening identified 264 mutant lines with substantially different HCNp levels, either lower or higher, compared to non-mutated parent lines. Leaf samples were taken from the selected plants (3-4 day old seedlings - "up to the 4th leaf" stage) and accurate levels of HCNp determined. Sixty-eight lines were identified with no or very low HCNp levels (Table 2) and 38 with very high HCN levels, confirming the HCNp levels seen for these lines by the FA paper screen. However, 32 mutant lines had undetectable HCNp levels by the FA paper screen but the quantifiable assay, which involves the addition of  $\beta$ -glucosidase, indicated that these lines had high HCNp levels. These lines are likely to be  $\beta$ -glucosidase mutants (Table 2; nb. they were not analysed further as ruminants such as cattle carry microorganisms in the rumen that have  $\beta$ -glucosidase activity capable of breaking down dhurrin, so mutations in the two  $\beta$ -glucosidase genes found in sorghum would have little agronomic benefit) (Blomstedt *et al.*, 2012).

### Identification and molecular modelling of mutations in CYP79A1

Genomic DNA was isolated from the 264 selected lines and analysed by TILLING to identify putative mutations in the CYP79A1 and UGT85B1 genes. The putative CYP79A1 mutant lines were analysed in detail and sequencing confirmed eleven independent mutant lines (Figure 2). All of the mutations were G/C-to-A/T transitions as expected following EMS treatments resulting in three lines with silent amino acid changes, the generation of a stop codon in one line and seven lines with amino acid changes (Table 3)(Blomstedt *et al.*, 2012)..

The position of the mutated residues was compared to a homology model of CYP79A1 (Figure 2B, Jensen *et al.*, 2011). Two of the mutations (E145K, A154T) are found in the proposed substrate-binding site and mutation of these residues directly alters the shape of the substrate pocket to affect the binding of tyrosine. The P414L mutation (found in line 2-908-1) is found 10.7 Å away from the substrate-binding site and, while not wishing to be bound by theory, it is consequently anticipated that this mutation would not directly interfere with binding of substrate (Figure 2B). Nevertheless,

this mutation is found at the end of helix K buried deeply inside the protein and replacement of the highly conserved proline residue at this site for a leucine is likely to perturb the substrate cavity and/or heme binding sufficiently to disrupt catalytic activity. Two mutations (M98I and E529K) are on the periphery of the enzyme and the enzyme's apparent inability to metabolise tyrosine to *p*-hydroxyphenylacetaldoxime is not easily explained in a structural context. Three additional mutations identified in the screen (R39stop, T48I and P60S) are all in the transmembrane anchor (which is presumably a non-catalytic domain of the enzyme), which is not part of the available homology model. The plant line carrying the R39stop mutation was very poor growing and set no seed in the M2 generation.

The mutant plant line 2-908-1 (containing a P414L mutation) was selected for further study based upon the anticipated effect of the mutation (ie to disrupt catalytic activity) and its almost undetectable levels of HCNp (both by FA and quantifiable HCNp assay) compared to non-mutated control lines which contained levels of dhurrin that were readily detectable using the FA paper and quantifiable HCNp assay technique. Mutant plant line 2-908-1 also exhibited good field growth and an apparently normal phenotype upon reaching maturity compared to non-mutated control plants. In addition, three mutant plant lines which were suspected to be regulatory mutants (ie 2-1307-2, 4-565-1 and 4-970-1; see Table 2), were also subjected to further study.

**Table 2 Classification of mutant plant lines**

| Gene mutated             | Phenotype                        | Number of lines |
|--------------------------|----------------------------------|-----------------|
| CYP79A1                  | Absence or reduction in dhurrin  | 9               |
| CYP79A1                  | Increase in dhurrin              | 2               |
| UGT85B1                  | Absence or reduced dhurrin       | 13              |
| $\beta$ -glucosidase 1/2 | No $\beta$ -glucosidase activity | 32              |
| Unknown                  | Regulatory mutant                | 3               |
| Unknown                  | Brown midrib (BMR)               | 7               |
| Unknown                  | High biomass and/or high sugar   | 2               |

**Table 3**

| <b>Mutant Line</b> | <b>Nucleotide Mutation</b> | <b>Translation</b>  | <b>Note</b>      |
|--------------------|----------------------------|---------------------|------------------|
| 2-141-1            | C/T                        | Stop codon 39       | No seed after M2 |
| 4-723-1            | C/T                        | Silent mutation 42  | No seed after M2 |
| 4-1406-1           | C/T                        | T48I                |                  |
| 4-314-3            | C/T                        | P60S                |                  |
| 2-2480-3           | C/T                        | Silent mutation 78  |                  |
| 5-150-1            | G/A                        | M98I                |                  |
| 5-84-1             | G/A                        | E145K               |                  |
| 5-132-1            | G/A                        | A154T               | No seed after M3 |
| 4-705-2            | C/T                        | Silent mutation 225 |                  |
| 2-908-1            | C/T                        | P414L               |                  |
| 4-327-2            | G/A                        | E529K               |                  |

#### 5 Dark grown plant material and microsomal preparations

The following mutant plant lines were germinated in the dark and microsomes prepared from the seedlings following germination for four days:

- (i) M4 lines of the selected 2-908-1 CYP79A1 mutant, 2-908-1-1-5 and 2-908-1-5-2;
- (ii) M4 lines of the suspected regulatory mutants (Table 2), 2-1307-2-10-1, 2-1307-2-10-5, 4-565-1-11-2 and 4-970-1-1-2, 4-970-1-6-1 and 4-970-1-10-3.

The germination rate of the seeds from all lines was high and the only clear phenotypic difference from wild-type seedlings was observed with line 2-908-1-5-2 which exhibited markedly shorter seedlings (Figure 4). Reduced seedling length was also observed in the line 2-908-1-1-5 but less marked compared to 2-908-1-5-2 (Figure 4).

The pathway for conversion of tyrosine into dhurrin is catalysed by two multifunctional cytochromes P450 (CYP79A1 and CYP71E1) and by a UDPG-dependent glucosyltransferase

- (UGT85B1). The catalytic activity of the P450s is dependent on electron transfer from NADPH-cytochrome P450 oxidoreductase (CPR; Jensen and Moller, 2010). The two P450s, as well as CPR, are membrane bound and are thus recovered in the microsomal preparation. Without an active CPR enzyme, the P450s would not show any activity. Because the EMS treatment might have affected CPR activity, the CPR activity was measured in wild-type lines and the individual TILLING lines. The activity of NADPH-cytochrome P450 oxidoreductase in each of the microsomal preparations was measured as its ability to reduce cytochrome *c* (Horseheart cytochrome *c*, Sigma-Aldrich, cat no 7752) as monitored spectrometrically at 550 nm. Sample cuvette (total volume: 1 mL) contained 50  $\mu$ L 1 mM cytochrome *c* and 5  $\mu$ L microsomal membrane preparation in 50 mM  $KP_i$  (pH 7.9). Following monitoring of the background reduction rate for 3 min, 10  $\mu$ L 62 mM NADPH was added and the initial rate of cytochrome *c* reduction was measured. No NADPH was added to the reference cuvette. At the assay conditions used, 1 nmol of NADPH-cytochrome P450 oxidoreductase will reduce about 3,000 nmol of cytochrome *c* per min enabling quantification of the activity in each sorghum seedling (Guengerich *et al.*, 2009). The parent line showed an activity of 3.1 nmol cytochrome *c* reduced per min per seedling. The activity of the TILLING lines varied in the range from 3.0 to 4.7 nmol cytochrome *c* reduced per min per seedling. Thus, none of the TILLING lines showed major differences in CPR activity and no line showed absence of CPR activity, which would have resulted in the inability of the P450s to carry out the catalytic cycle.
- The CYP79A1 and CYP71E1 activity of the microsomal membranes was monitored by the ability to metabolise radio-labelled tyrosine. Two TILLING lines (2-908-1-1-5 and 2-908-1-5-2) were completely devoid of CYP79A1 activity resulting in no metabolism of the radio-labelled tyrosine. At saturating substrate concentrations, the line 2-1307-2-10-5 showed an 80% reduction in activity whereas 4-565-1-11-2 and 4-970-1-10-3 showed a 20% reduction of activity. In none of the TILLING lines did *p*-hydroxyphenylacetaldoxime or *p*-hydroxyphenylacetonitrile accumulate, indicating that the activity of CYP71E1 was not limiting for the conversion of intermediates into *p*-hydroxymandelonitrile, which is the final product of the dhurrin pathway obtained when membrane bound enzymes of microsomal preparations are used as the source.
- When the dhurrin content of the wild-type and TILLING lines was analysed, two mutant plant lines (2-908-1-1-5 and 2-908-1-5-2) were found not to accumulate any dhurrin. This matched the inability of the microsomal preparations isolated from these two plants to exhibit any CYP79A1 activity. The dhurrin content of the other lines varied from a level similar to that of wild-type lines to twice the wild-type level per seedling. There was no direct correlation between the dhurrin

content and the reduced activity of CYP79A1 measured in lines 2-1307-2-10-5, 4-565-1-11-2 and 4-970-1-10-3 (putative regulatory mutants with negligible dhurrin in mature leaf tissue). However, the amount of accumulated dhurrin reflects the proportion between the rate of synthesis and turnover. It is known that in wild-type seedlings, the dhurrin content of dark grown seedlings does start to decrease after 4 days, so a direct relationship between CYP79A1 activity and dhurrin content is not to be expected.

Accordingly, three mutant plant lines (2-1307-2, 4-970-1 and 4-565-1), that were initially identified (both by FA and quantifiable HCNp assay) as being acyanogenic or having very low HCNp levels, were subsequently shown to have very high levels of HCNp in etiolated seedlings (ie from the assays of the microsomal membrane preparations). Subsequent analysis of HCNp in whole seedlings during plant growth (up to the 4th leaf stage) showed that, in the 2-1307-2 and 4-970-1 lines, the HCNp drops to very low HCNp levels quickly and, as such, these lines may harbour a mutation in a regulatory gene. The mutant plant line 4-565-1 does not drop as rapidly, but additional preliminary experiments indicate that the HCNp is only found within sheath tissue and not leaf tissue (*data not shown*), suggesting that this line may be a cyanogenic glucoside transport mutant. Moreover, the three genes encoding CYP79A1, CYP71E1 and UGT85B1, have been sequenced in each of these lines and no mutations were identified.

#### Growth and HCNp characteristics of 2-908-1 mutant M4 lines

Despite the observed shorter seedling length of 2-908-1 mutant lines, these plants showed good growth characteristics in the field and greenhouse (Figure 5). Further, mature plants showed an apparently normal phenotype. Moreover, nitrate analysis indicated that the mutation contained in these lines does not result in an increase in nitrate in these plants (*data not shown*).

Sequencing confirmed that representative 2-908-1 M4 lines, namely 2-908-1-1-5 and 2-908-1-5-2, are homozygous for the mutation resulting in the P414L amino acid substitution. In assays of HCNp of line 2-908-1 through M2 to M4, it was found that in most cases the M4 plants showed almost undetectable levels of HCNp compared to non-mutated control plants (Figure 6). The exceptions were lines 2-908-1-3-2, 2-908-1-4-1, 2-908-1-4-3 and 2-908-1-4-4 which were high in HCNp. This indicates that it is likely the M3 plants 2-908-1-3 and 2-908-1-4 were heterozygous for the P414L mutation (Figure 6).

#### Northern analysis of 2-908-1 mutant M4 lines

Northern analysis of several 2-908-1 mutant M4 lines showed that the CYP79A1 transcript is present in all individuals at a relatively uniform but relatively low level of expression.

#### 5 Immunoblotting of the sorghum TILLING lines

Antibodies synthesised to a CYP79A1 peptide (14 amino acids) was shown to be specific to CYP79A1. Microsomal proteins were run on a 12% polyacrylamide gel, transferred to nitrocellulose and probed with the CYP79A1 specific antibody. This detected the CYP79A1 protein in all CYP79A1-mutated plant lines indicating that despite the respective mutations, the  
10 plants were still able to produce a CYP79A1 protein; only that it is non-functional.

#### *Discussion*

A number of mutant sorghum plant lines were generated that showed reduced HCNp. One particular mutant line (2-908-1) was selected based upon the anticipated effect of the mutation (ie  
15 to disrupt catalytic activity), the capacity for good field growth with an apparently normal phenotype at maturity and almost undetectable levels of HCNp (both by FA and quantifiable HCNp assay) compared to non-mutated control lines which did possess HCNp. M5 seed of mutant line 2-908-1 (particularly, offspring derived from the selfing of 2-908-1-5-4) was deposited with the National Collections of Industrial, Food and Marine Bacteria (NCIMB) under the *Budapest Treaty*  
20 on 14 April 2011, Accession No 41826. Plants of this mutant line are particularly suitable for use as novel stock feed crop plants. In response to poor public perception and market resistance to GMOs, the plants described in this example have been generated without the use of techniques of genetic manipulation. As such, plants and plant cells according to the present invention preferably comprise a mutation that has originated by mutagenesis (eg by treatment with a mutagenic agent or  
25 mutagenic radiation) rather than by genetic manipulation or spontaneous natural mutation.

Throughout this specification the word "comprise", or variations such as "comprises" or  
30 "comprising", will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

All publications mentioned in this specification are herein incorporated by reference. Any discussion of documents, acts, materials, devices, articles or the like which has been included in the present specification is solely for the purpose of providing a context for the present invention. It is not to be taken as an admission that any or all of these matters form part of the prior art base or  
5 were common general knowledge in the field relevant to the present invention as it existed in Australia or elsewhere before the priority date of each claim of this application.

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the  
10 spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

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**CLAIMS**

1. A plant cell of, or derived from, a plant species that, in wild-type, produces cyanogenic dhurrin, wherein the plant cell comprises a mutation in one or more genes encoding the enzymes of the biosynthetic pathway for production of said dhurrin in said plant cell, such that said plant cell either can not produce dhurrin or produces or accumulates dhurrin in low amounts relative to a corresponding plant cell of said wild-type.

2. The plant cell of claim 1, wherein the plant cell is of a sorghum species.

3. The plant cell of claim 1 or 2, wherein the plant cell forms part of a whole plant.

4. The plant cell of any one of claims 1 to 3, wherein the mutation is located in the gene of cytochrome P450 CYP79A1, cytochrome P450 CYP71E1 or UDP-glucosyltransferase UGT85B1.

5. The plant cell of any one of claims 1 to 3, wherein the mutation is located in the gene of cytochrome P450 CYP79A1.

6. The plant cell of any one of claims 1 to 5, wherein the plant cell is homozygous for the mutation.

7. A sorghum plant or hybrid which comprises a mutation in a gene encoding cytochrome P450 CYP79A1, cytochrome P450 CYP71E1 or UDP-glucosyltransferase UGT85B1 which renders the gene defective in that it is not expressed or otherwise encodes an enzyme with no or diminished activity, such that said plant either can not produce dhurrin or produces or accumulates dhurrin in low amounts relative to a corresponding plant cell of said wild-type.

8. The sorghum plant or hybrid of claim 7, wherein the plant or hybrid comprises a mutation in a gene encoding cytochrome P450 CYP79A1 which renders the gene defective in that it is not expressed or otherwise encodes a CYP79A1 enzyme with no or diminished activity.

9. The sorghum plant or hybrid of claim 8, wherein the plant or hybrid comprises the mutated CYP79A1 gene of the mutant plant line 2-908-1-5-4 (NCIMB Accession No 41826).

10. A sorghum plant of mutant plant line 2-908-1-5-4 (NCIMB Accession No 41826).
11. A plant of, or derived from, a plant species that, in wild-type, produces cyanogenic dhurrin, wherein the plant comprises a mutation in one or more genes encoding the enzymes of the biosynthetic pathway for production of said dhurrin, which thereby provides the plant with an acyanogenic or low HCNp trait as shown by mutant plant line 2-908-1-5-4, wherein representative seeds of which have been deposited with the NCIMB under Accession No 41826.
12. A plant cell of the plant according to claim 11.
13. A mutant polynucleotide molecule comprising;
  - (i) a nucleotide sequence comprising a defective regulatory region of a gene encoding cytochrome P450 CYP79A1, cytochrome P450 CYP71E1 or UDP-glucosyltransferase UGT85B1, and/or
  - (ii) a nucleotide sequence encoding a truncated or otherwise mutated cytochrome P450 CYP79A1, cytochrome P450 CYP71E1 or UDP-glucosyltransferase UGT85B1, wherein said polynucleotide molecule is in a substantially isolated form.
14. A vector including a mutant polynucleotide molecule comprising;
  - (i) a nucleotide sequence comprising a defective regulatory region of a gene encoding cytochrome P450 CYP79A1, cytochrome P450 CYP71E1 or UDP-glucosyltransferase UGT85B1, and/or
  - (ii) a nucleotide sequence encoding a truncated or otherwise mutated cytochrome P450 CYP79A1, cytochrome P450 CYP71E1 or UDP-glucosyltransferase UGT85B1.
15. The polynucleotide molecule of claim 13, comprising;
  - (i) a nucleotide sequence comprising a defective regulatory region of a gene encoding cytochrome P450 CYP79A1 enzyme, and/or
  - (ii) a nucleotide sequence encoding a truncated or otherwise mutated cytochrome P450 CYP79A1 enzyme, wherein said polynucleotide molecule is in a substantially isolated form.
16. The polynucleotide molecule of claim 13, comprising a nucleotide sequence substantially corresponding to that of the mutated CYP79A1 gene of the mutant plant line 2-908-1-5-4 (NCIMB Accession No 41826).

17. The vector of claim 14 including a mutant polynucleotide molecule comprising;
  - (i) a nucleotide sequence comprising a defective regulatory region of a gene encoding cytochrome P450 CYP79A1 enzyme, and/or
  - (ii) a nucleotide sequence encoding a truncated or otherwise mutated cytochrome P450 CYP79A1 enzyme.
18. The vector of claim 14, wherein the polynucleotide molecule comprises a nucleotide sequence substantially corresponding to that of the mutated CYP79A1 gene of the mutant plant line 2-908-1-5-4 (NCIMB Accession No 41826).
19. Harvested grain or straw of a plant according to any one of claims 7 to 11.

1/4

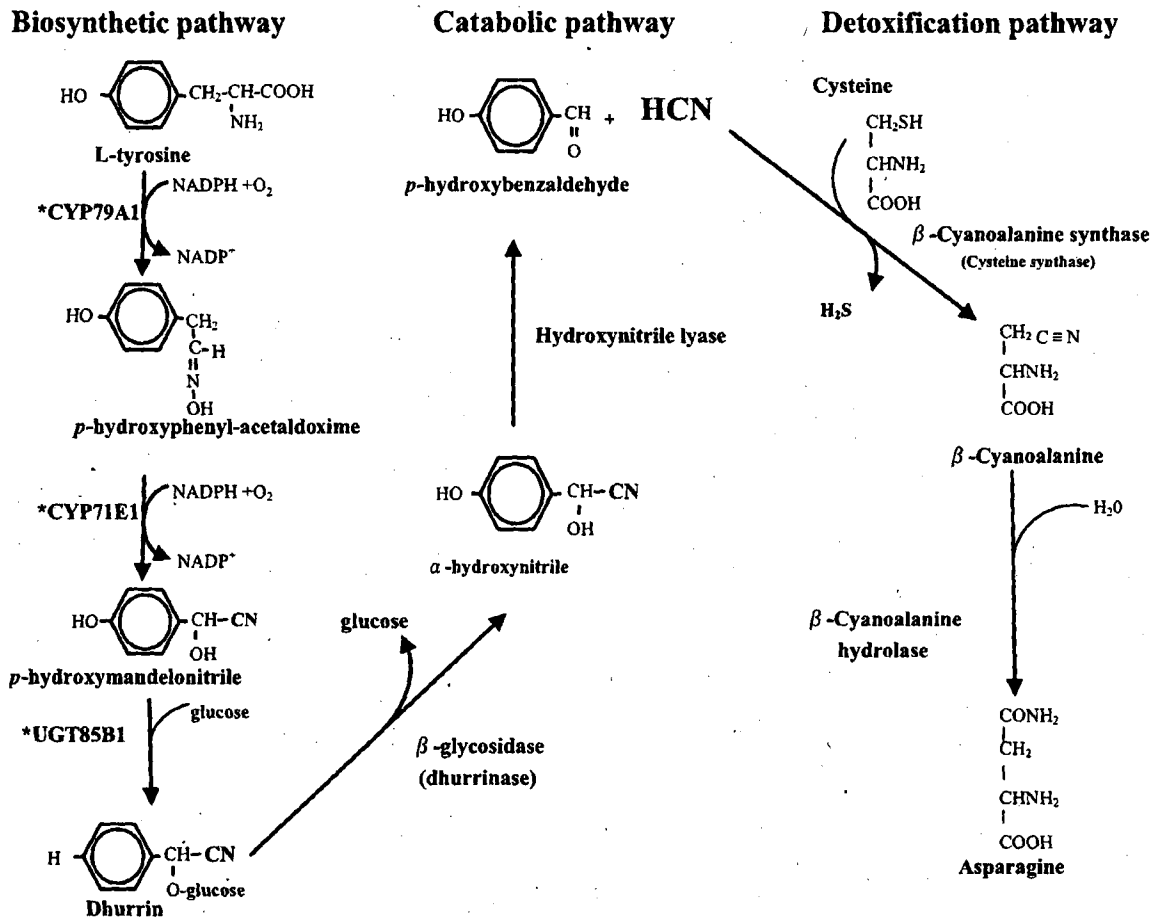


Figure 1

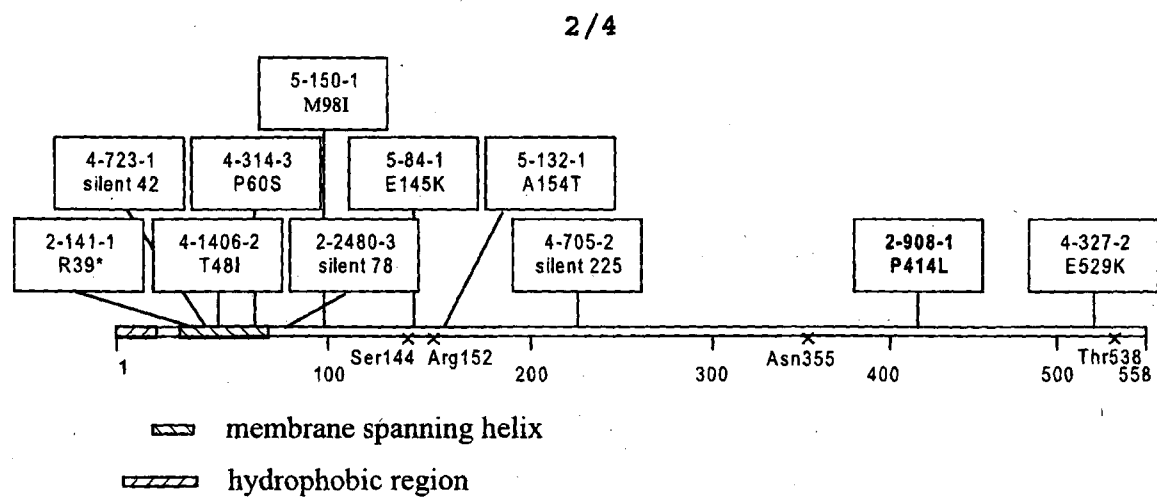


Figure 2A

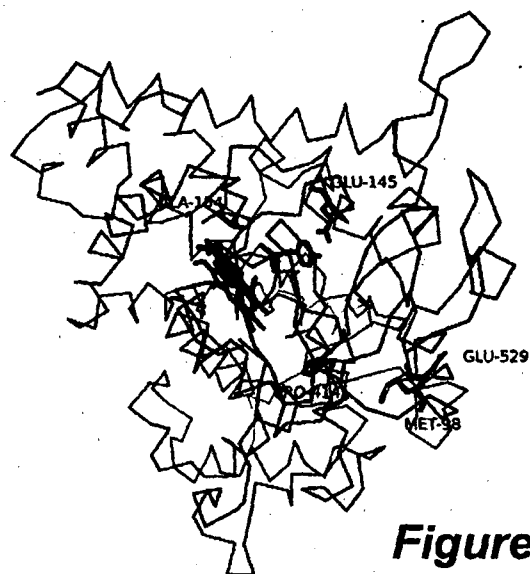


Figure 2B

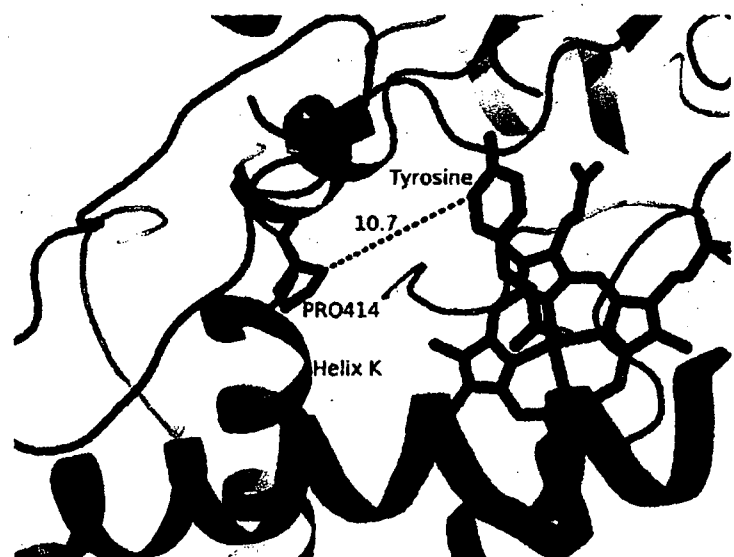
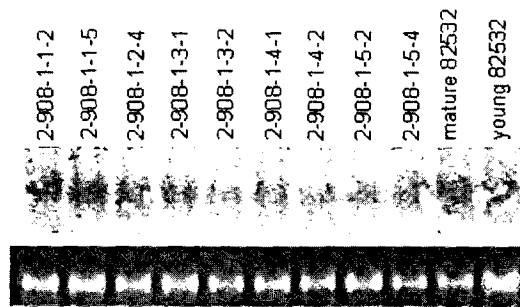
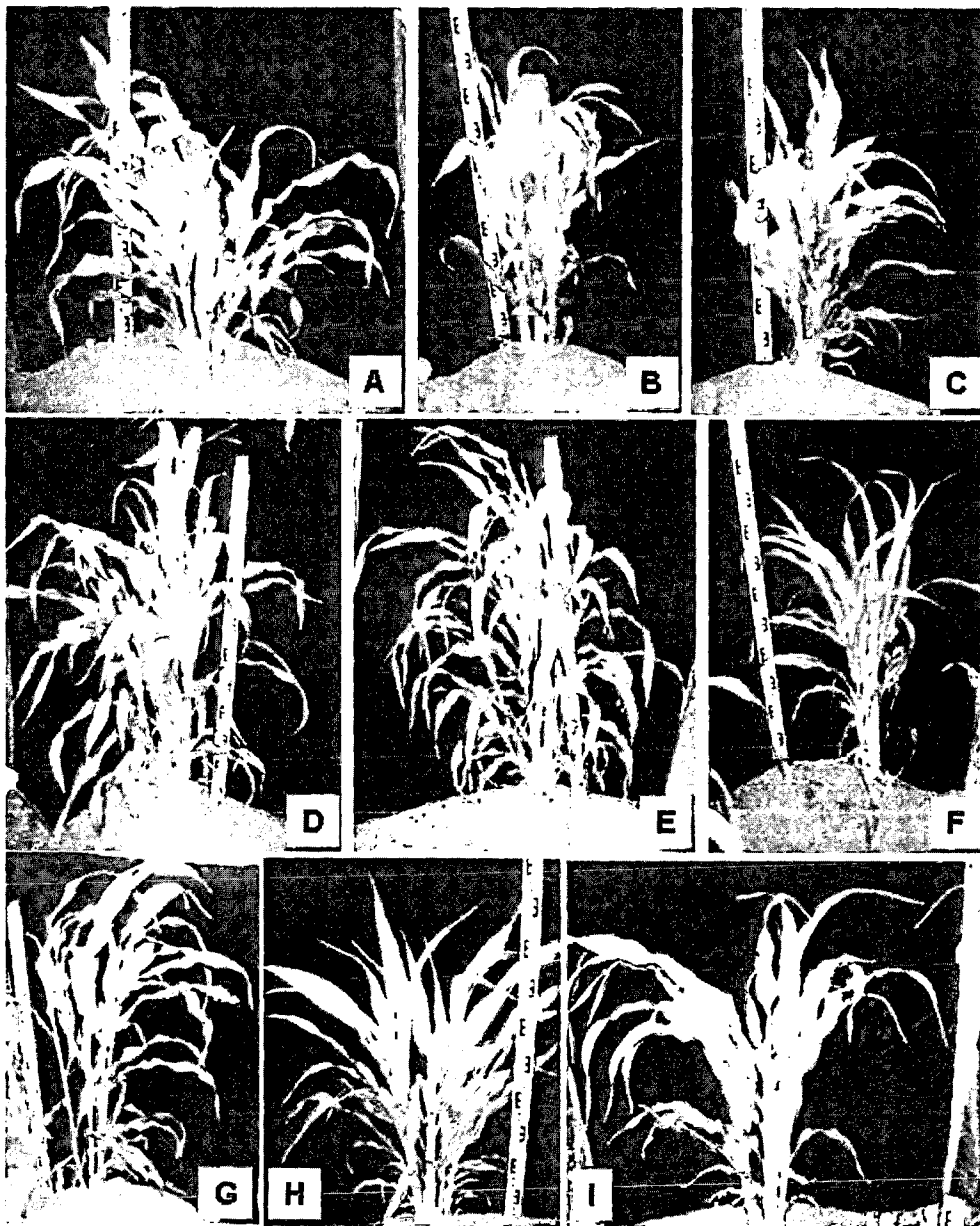


Figure 2C

3/4

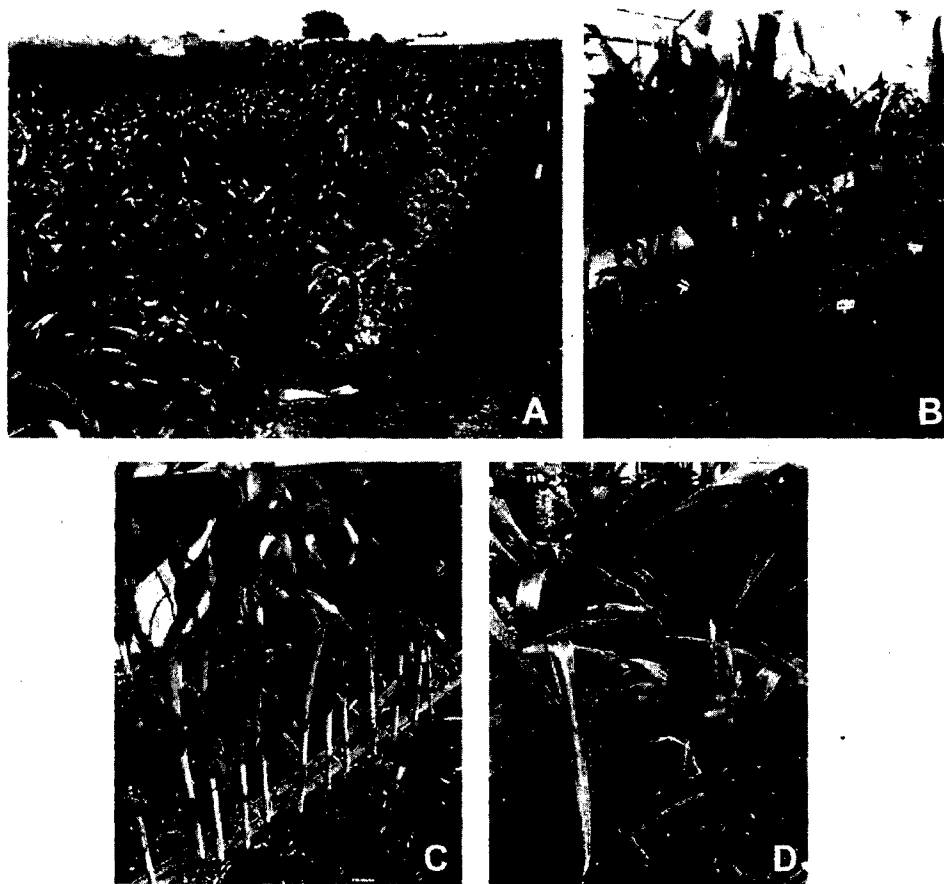


**Figure 3**

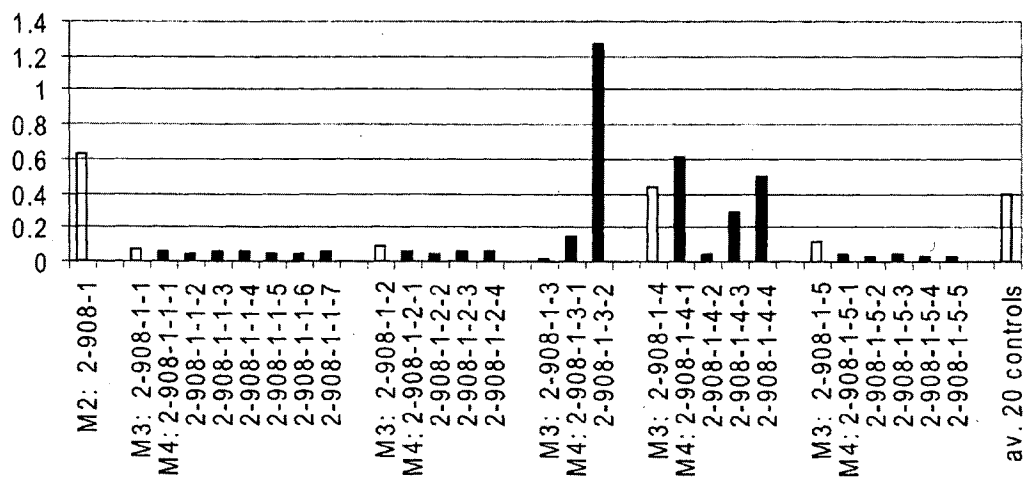


**Figure 4**

4/4



**Figure 5**



**Figure 6**

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/AU2012/000298

|                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                              |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                          |                                                                                                                                                   |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                        |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                          |  |
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| <b>A. CLASSIFICATION OF SUBJECT MATTER</b><br>Int. Cl.<br><i>A01H 5/00</i> (2006.01) <i>C12N 15/113</i> (2010.01) <i>C12N 15/29</i> (2006.01)<br>According to International Patent Classification (IPC) or to both national classification and IPC                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                           |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                          |                                                                                                                                                   |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                        |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                          |  |
| <b>B. FIELDS SEARCHED</b><br>Minimum documentation searched (classification system followed by classification symbols)<br>Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched<br>Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)<br>WPI, EPODOC, MEDLINE, HCPALUS, AGRICOLA, BIOSIS & keywords: dhurrin, sorghum, CYP79A1, CYP71E1, UGT85B1, mutation, reduced, 2-908-1-5-4 and like terms.                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                           |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                          |                                                                                                                                                   |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                        |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                          |  |
| <b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                          |                                                                                                                                                   |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                        |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                          |  |
| Category*                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                    | Citation of document, with indication, where appropriate, of the relevant passages                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                       | Relevant to claim No.                                                                                                                             |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                        |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                          |  |
| X                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                            | WO 1998/040470 A2 (NOVARTIS AG et al.) 17 September 1998<br>See pages 3, 4, 8, 15                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                        | 1-8, 11-19                                                                                                                                        |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                        |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                          |  |
| X                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                            | WO 2001/040491 A2 (LUMINIS PTY, LIMITED et al.) 7 June 2001<br>See pages 11, 12                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                          | 1-8, 11-19                                                                                                                                        |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                        |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                          |  |
| X                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                            | WO 2001/051622 A2 (SYNGENTA PARTICIPATIONS AG et al.) 19 July 2001<br>See pages 18, 26-32                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                | 13-18                                                                                                                                             |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                        |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                          |  |
| <input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C <input checked="" type="checkbox"/> See patent family annex                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                    |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                          |                                                                                                                                                   |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                        |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                          |  |
| <table style="width: 100%; border: none;"> <tr> <td style="width: 33%; vertical-align: top;">           * Special categories of cited documents:<br/>           "A" document defining the general state of the art which is not considered to be of particular relevance<br/>           "E" earlier application or patent but published on or after the international filing date<br/>           "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)<br/>           "O" document referring to an oral disclosure, use, exhibition or other means<br/>           "P" document published prior to the international filing date but later than the priority date claimed         </td> <td style="width: 33%; vertical-align: top;">           "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention<br/>           "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone<br/>           "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art<br/>           "&amp;" document member of the same patent family         </td> <td style="width: 33%;"></td> </tr> </table> |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                          |                                                                                                                                                   | * Special categories of cited documents:<br>"A" document defining the general state of the art which is not considered to be of particular relevance<br>"E" earlier application or patent but published on or after the international filing date<br>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)<br>"O" document referring to an oral disclosure, use, exhibition or other means<br>"P" document published prior to the international filing date but later than the priority date claimed | "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention<br>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone<br>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art<br>"&" document member of the same patent family |  |
| * Special categories of cited documents:<br>"A" document defining the general state of the art which is not considered to be of particular relevance<br>"E" earlier application or patent but published on or after the international filing date<br>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)<br>"O" document referring to an oral disclosure, use, exhibition or other means<br>"P" document published prior to the international filing date but later than the priority date claimed                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                       | "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention<br>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone<br>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art<br>"&" document member of the same patent family |                                                                                                                                                   |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                        |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                          |  |
| Date of the actual completion of the international search<br>19 April 2012                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                   |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                          | Date of mailing of the international search report<br>23 April 2012                                                                               |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                        |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                          |  |
| Name and mailing address of the ISA/AU<br>AUSTRALIAN PATENT OFFICE<br>PO BOX 200, WODEN ACT 2606, AUSTRALIA<br>E-mail address: pct@ipaustalia.gov.au<br>Facsimile No. +61 2 6283 7999                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                        |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                          | Authorized officer<br><b>RICHARD FILMER</b><br>AUSTRALIAN PATENT OFFICE<br>(ISO 9001 Quality Certified Service)<br>Telephone No : +61 2 6283 2735 |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                        |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                          |  |

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/AU2012/000298

| C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT |                                                                                                                                                                                                                                                                                                                                                                                                                     |                       |
|-------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------|
| Category*                                             | Citation of document, with indication, where appropriate, of the relevant passages                                                                                                                                                                                                                                                                                                                                  | Relevant to claim No. |
| X                                                     | THORSØE, K. S. et al., "Determination of Catalytic Key Amino Acids and UDP Sugar Donor Specificity of the Cyanohydrin Glycosyltransferase UGT85B1 from <i>Sorghum bicolor</i> . Molecular Modeling Substantiated by Site-Specific Mutagenesis and Biochemical Analyses", Plant Physiology, 2005, vol. 139, pages 664-673<br>See Abstract; Materials and Methods: Construction and Analysis of Site-Directed Mutants | 13, 14                |
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**INTERNATIONAL SEARCH REPORT**

Information on patent family members

International application No.

**PCT/AU2012/000298**

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

| Patent Document Cited in<br>Search Report                                                                                 |    | Patent Family Member |    |            |               |
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| Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001. |    |                      |    |            |               |
| END OF ANNEX                                                                                                              |    |                      |    |            |               |