The biosynthetic gene cluster for the cyanogenic glucoside dhurrin in *Sorghum bicolor* contains its co-expressed vacuolar MATE transporter

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Genomic gene clusters for the biosynthesis of chemical defence compounds are increasingly identified in plant genomes. We previously reported the independent evolution of biosynthetic gene clusters for cyanogenic glucoside biosynthesis in three plant lineages. Here we report that the gene cluster for the cyanogenic glucoside dhurrin in *Sorghum bicolor* additionally contains a gene, *SbMATE2*, encoding a transporter of the multidrug and toxic compound extrusion (MATE) family, which is co-expressed with the biosynthetic genes. The predicted localisation of *SbMATE2* to the vacuolar membrane was demonstrated experimentally by transient expression of a *SbMATE2*-YFP fusion protein and confocal microscopy. Transport studies in *Xenopus laevis* oocytes demonstrate that *SbMATE2* is able to transport dhurrin. In addition, *SbMATE2* was able to transport non-endogenous cyanogenic glucosides, but not the anthocyanin cyanidin 3-O-glucoside or the glucosinolate indol-3-yl-methyl glucosinolate. The genomic co-localisation of a transporter gene with the biosynthetic genes producing the transported compound is discussed in relation to the role self-toxicity of chemical defence compounds may play in the formation of gene clusters.

Plants produce a wide variety of chemical defence compounds that provide protection against herbivores and pathogens. A particular plant species or genus is characterised by the presence of a subset of such defence compounds. Considerable inter- and intraspecific variation is thought to result from various trades-offs, such as between growth and defence, or between competing defence strategies in a varying ecological context. Besides constraints on the allocation of resources, chemical defence also carries the risk of self-toxicity as a metabolic cost. One specific class of chemical defence compounds are the cyanogenic glucosides, which occur widely in the plant kingdom. These compounds are glucosides of amino acid derived α-hydroxynitriles, and part of a two-component chemical defence system. Hydrolysis of cyanogenic glucosides by a specific β-glucosidase following tissue disruption, for instance by chewing insects, releases the chemically unstable α-hydroxynitrile, which upon dissociation gives rise to the formation of toxic hydrogen cyanide. The cyanogenic glucoside dhurrin is the main chemical defence compound in *Sorghum bicolor*, an important cereal crop used for food and feed. Dhurrin is derived from the amino acid tyrosine by the sequential action of two cytochrome P450 enzymes, named CYP79A1 (Sobic.001G012300) and CYP71E1 (Sobic.001G012200), and the glucosylation and stabilisation of the produced α-hydroxynitrile (cyanohydrin) intermediate by the UDP-glucosyltransferase UGT85B1 (Sobic.001G012400). As it involves two membrane anchored cytochrome P450 enzymes, dhurrin biosynthesis is thought to take place at the cytosolic surface of the endoplasmic reticulum (ER). The glucosylated compound,
which is labile in non-acidic environments due to the ionization of the hydroxyl group on the benzene ring, is stably stored in the acidic vacuolar compartment but the mechanism of its intracellular transport from the ER to the vacuole is unknown.

The biosynthetic pathways for cyanogenic glucosides have also been elucidated in dicot plant species such as cassava (Manihot esculenta) and the model legume Lotus japonicus. Gene identification in L. japonicus was helped by the fact that the biosynthetic genes were found to be co-localised in the same genome region, and in this species the second step was catalysed by a member of the CYP736 gene family. The biosynthetic genes in cassava and sorghum were also found to be organised in a gene cluster, but the three clusters are thought to have evolved independently. This remarkable genomic co-localisation of non-homologous genes encoding biosynthetic enzymes in the same metabolic pathway has also been observed for other classes of plant chemical defence compounds such as terpenoids, benzoxazinoids, and alkaloids. These clusters are proposed to promote the co-inheritance of beneficially interacting alleles and to additionally facilitate the co-expression of the biosynthetic genes by regulation at the chromatin level. An important driver for gene cluster formation and maintenance, via selection for reduced recombination between the interacting genes, is thought to be the fact that incompletely inherited biosynthetic pathways may result in the release of toxic intermediates causing self-toxicity.

Membrane transport is increasingly recognised as an important component of plant specialised metabolism and bioengineering approaches, but the number of characterised transporters remains limited. Members of the large multidrug and toxic compound extrusion (MATE) gene family are found in both prokaryotes and eukaryotes, and transport a wide range of compounds. In plants they have been shown to transport xenobiotic compounds, organic acids, plant hormones, and secondary metabolites such as anthocyanins and other flavonoids, and the alkaloid nicotine. Here we report that the biosynthetic gene cluster for dhurrin additionally includes a gene encoding a tonoplast localised MATE transporter for dhurrin uptake, demonstrating that the analysis of plant gene clusters can contribute to transporter identification.

**Results and Discussion**

The dhurrin gene cluster. Analysis of the genomic region surrounding the dhurrin biosynthetic gene cluster in sorghum, revealed the presence of genes encoding a MATE transporter (Sobic.001G12600) we have named SbMATE2, and a glutathione S-transferase (GST) named SbGST1 (Sobic.001G12500) of the plant specific phi subfamily (Fig. 1a). Additional support for the involvement of these two genes in dhurrin metabolism was their co-expression with the biosynthetic genes, as revealed by searching the MOROKOSHI sorghum transcriptome database containing publicly available RNA-seq data. The genes showing the highest co-expression with CYP79A1, encoding the first enzyme of the dhurrin biosynthetic pathway, were CYP71E1, immediately followed by SbMATE2 (Fig. 1b, Supplementary Table 1). Co-expression with CYP79A1 was additionally observed for the SbGST1 and the UGT85B1 genes, which showed the highest level of co-expression with each other. High relative expression of all genes was observed in shoots of 9-day old seedlings (Fig. 1b, condition 16), which was enhanced by abscisic acid and osmotic stress treatments (conditions 14 and 15, respectively). Co-expression studies have resulted in transporter identification, as was reported for several MATE vacuolar nicotine transporters in Nicotiana tabacum. Moreover, the co-expression of clustered biosynthetic genes from the same pathway was reported for the synthesis of the triterpene thalianol in Arabidopsis thaliana, and such tight coordinated regulation is suggested to prevent the accumulation of deleterious biosynthetic intermediates. This toxicity argument also applies to the labile and reactive nature at cytoplasmic pH. The coordinated expression of genes in transport or storage with those of the biosynthesis pathway, is similarly proposed to reduce or prevent the self-toxic effects of the metabolites produced.
GSTs are well known for conjugating the tripeptide glutathione to endogenous toxic products and xenobiotic compounds, but also for non-enzymatic roles as carrier proteins for endogenous reactive molecules such as porphyrins and anthocyanins. Like in the biosynthesis of cyanogenic glucosides, anthocyanins are produced by cytochrome P450 enzymes on the cytoplasmic surface of the endoplasmic reticulum and it is of particular interest to note that anthocyanin transport to the vacuole requires the action of both a GST and a MATE transporter. This is for instance demonstrated by the transparent testa mutants in Arabidopsis, where the TT19 gene encodes a GST, and where TT12 encodes a MATE transporter. The precise mechanism of anthocyanin transport is the subject of much debate, may involve vesicle mediated trafficking, and was suggested to be related to the removal of toxic compounds from the cytoplasm. Although similar mechanisms may have been recruited in the case of dhurrin, a potential role for SbGST1 in dhurrin metabolism remains to be established and could be indirect, such as in dealing with the cellular effects of dhurrin self-toxicity.

SbMATE2 is localised to the vacuolar membrane. The sequence of the SbMATE2 transporter gene was experimentally verified by cDNA cloning from seedlings and shown to contain two small introns, 135 bp and 97 bp in length respectively, positioned in the C-terminal half of the protein coding region. The transcript encodes...
a 498 amino acid polypeptide predicted by the Phyre2 web portal for protein modelling to show the topology of the twelve transmembrane helixes typical for prokaryotic and plant MATE transporters34–36 (Supplemental Fig. 1).

The structural model based on the NorM transporter from *Vibrio cholerae*, and an additional amino acid sequence alignment that includes mammalian MATE transporters, indicated that SbMATE2 contains conserved amino acids that are part of the cation-binding motif reported for NorM-VC35,37 (Supplemental Fig. 2). The functional-ity of a predicted N-terminal tonoplast targeting signal was experimentally investigated by transiently expressing a SbMATE2-YFP fusion protein in *Nicotiana benthamiana* followed by confocal microscopy. Co-expression of SbMATE2-YFP with either one of two aquaporin based organelle specific markers was used to distinguish between the tonoplast and plasma membrane38. SbMATE2-YFP co-localised with the vacuolar membrane marker γ-TIP-CFP, a C-terminal fusion of CFP to full-length γ-TIP, as both signals were observed at positions where the tonoplast was not localised directly adjacent to the cell wall (Fig. 2a). In contrast, the AtPIP2A-CFP marker, consisting of the CFP fused to the plasma membrane aquaporin AtPIP2A, followed the cellular outline precisely (Fig. 2b). Tobacco protoplasts expressing the SbMATE2-YFP construct were used to further exclude a plasma membrane localisation of SbMATE2. Confocal microscopy showed that the SbMATE2-YFP fusion protein was only localised to the vacuolar membrane as it folds around the chloroplasts on the side internal to the cell (Fig. 2c).

**SbMATE2 transports cyanogenic glucosides.** Phylogenetic analysis placed SbMATE2 in what Shitan et al. designated as clade I, consisting of MATE transporters that are functionally characterised (Fig. 3)39. Most of the MATE transporters in this clade function in the accumulation of plant specialised metabolites such as flavonoids and alkaloids, perhaps also a reflection of the experimental interest in transporters for these compounds. The clade includes the seed coat expressed vacuum anthocyanin transporter AtTT1221,40, MtMATE1 and MtMATE2 from *Medicago truncatula* transporting flavonoid glucosides (and in the case of MtMATE2 also flavonoid glyco-side malonates)30, VvAM1 and VvAM3 from *Vitis vinifera* transporting acylated anthocyanins22, and the tobacco NtMATE1, NtMATE2, and Nt-JAT2 transporters for the vacuum sequestration of nicotine in leaves or roots of *Nicotiana tabacum*32,24.

**Figure 3.** In a phylogenetic analysis SbMATE2 is part of a clade containing MATE transporters for flavonoids and alkaloids. A molecular phylogenetic analysis was performed using the Maximum Likelihood method based on the Jones-Taylor-Thornton (JTT) matrix-based model for amino acid sequences. Branch lengths are measured in the number of substitutions per site, and positions containing gaps were eliminated. Bootstrap values (1000x) are indicated at branch points. Analyses were conducted using the MEGA5 software package47. The amino sequences used in the analysis were: SbMATE2 (*Sorghum bicolor*, Sobic.001G012600), OsPEZ1 (*Oryza sativa*, Os03g037490), OsPEZ2 (*O. sativa*, Os03g057290), OsMATE1 (*O. sativa*, Os03g08900), TT12 (*Arabidopsis thaliana*, At3g59030), FFT (*A. thaliana*, At4g25840), MdMATE1 (*Malus domestica*, GU64954), MdMATE2 (*M. domestica*, GU064956), MtMATE1 (*Medicago truncatula*, FJ858726), MtMATE2 (*M. truncatula*, HM856605), VvAM1 (*Vitis vinifera*, Fj264202), VvAM3 (*V. vinifera*, Fj264203), NtMATE1 (*Nicotiana tabacum*, AB286961), NtMATE2 (*N. tabacum*, AB286962), Nt-JAT1 (*N. tabacum*, AM991692), Nt-JAT2 (*N. tabacum*, AB922128), and ZmMATE2 (*Zea mays*, Fj873684). Coloured circles represent the transported compound classes: red = flavonoids, blue = alkaloids, purple = hydroxynitrile glucosides.
The possible role of SbMATE2 in dhurrin transport was studied by export experiments conducted in *Xenopus laevis* oocytes. Following injection of dhurrin and a 90 min incubation, SbMATE2 expressing oocytes showed an approximate 60% reduction in dhurrin content in comparison with oocytes not expressing SbMATE2, indicating dhurrin transport activity (Fig. 4). The SbMATE2 transporter was additionally able to transport the structurally related aromatic cyanogenic glucosides prunasin and the diglucoside amygdalin, the leucine derived cyanogenic glucoside epideridendrin, the non-cyanogenic \( \beta \)-hydroxynitrile glucoside epidermin, indol-3-yl-methyl glucosinolate (I3M), and the anthocyanin cyanidin 3-O-glucoside (C3G).

Our results demonstrate the presence of a non-biosynthetic component, the *SbMATE2* gene encoding a vacuolar transporter for dhurrin, in the gene cluster for a plant chemical defence pathway. Its inclusion in the dhurrin biosynthetic gene cluster is consistent with ideas that selection for reduced recombination between beneficially interacting alleles leads to gene cluster formation\(^{15,16,41}\). Such selection is proposed to result from antagonistic selection pressures, such as the benefits maintaining a functional pathway provides in specific ecological context, e.g. the presence of non-adapted herbivores, against the trade-off costs associated with it\(^{1,15}\). One of the negative cost associated with the production of chemical defence metabolites is the possibility of self-toxicity. Co-inheritance of a co-adapted gene set is thought to provide protection against the self-toxic biochemical nature of many chemical defence compounds or their pathway intermediates\(^{15,16}\). In the case of dhurrin the transport of this pH-dependent unstable cyanogenic glucoside from its cytoplasmic site of production to the acidic vacuole likely contributes to reducing self-toxicity. We previously also reported independently evolved biosynthetic gene clusters for cyanogenic glucosides in cassava and Lotus\(^8\). The main cyanogenic compounds produced by these species are linamarin and lotaustralin, respectively, which are more stable than dhurrin. In the most recent version of the cassava genome (*Manihot esculenta* v6.1), a MATE encoding gene named Manes.12G129000.1, which is not orthologous to *SbMATE2*, is present at a distance of about 325 kb of the described gene cluster. This physical linkage results in a high level of co-inheritance of Manes.12G129000.1 with the biosynthetic genes, but its physiological role is presently uncharacterised. The incomplete draft of the *Lotus japonicus* genome does not contain a transporter gene on the sequence contig that contains the biosynthetic gene cluster, but genetics has positioned at least one additional biosynthetic gene in hydroxynitrile glucoside metabolism in the vicinity of the gene cluster\(^8\). Eukaryotic biosynthetic gene clusters have been studied more extensively in fungi, and it is of interest to note that the inclusion of transporters is not uncommon in fungal gene clusters. The *TRI12* gene in *Fusarium sporotrichioides* is part of the biosynthetic gene cluster for terpene-derived trichothecene mycotoxins and encodes a trichothecene efflux pump\(^9\). Its disruption results in reduced growth and reduced levels of trichothecene production. A clear role in self-protection was reported for the *TOXA* gene in the fungal pathogen *Cochliobolus carbonum*, encoding...
an HC-toxin efflux pump essential for strains producing this toxic cyclic tetrapeptide. Fungi also contain metabolic gene clusters which provide nutritional benefits under certain ecological conditions. For example, the DAL cluster in Saccharomyces cerevisiae allows the use of allantoin, a degradation product of purines, as a nitrogen source instead of urate; providing an advantage in oxygen-poor environments. Apart from the catabolic genes, the DAL cluster also contains the DLAP gene encoding an allantoin permease. Given these examples from fungi, it can be expected that the future detailed analysis of genomic regions containing gene clusters for plant specialised metabolites will contribute to the identification of additional non-biosynthetic pathway components such as regulators or transporters.

Methods

Plant material, cDNA isolation and expression constructs. Total RNA was extracted from 3-day old etiolated seedlings of Sorghum bicolor. Following cDNA synthesis, a full length cDNA clone of SbMATE2 was amplified, cloned using the Zero Blunt TOPO PCR Cloning Kit (Invitrogen), and its sequence was verified. For localisation studies, the SbMATE2 protein coding region was amplified from the cDNA clone and fused in frame to YFP using USER cloning as described. The SbMATE2-YFP construct, under control of the 35S-CaMV promoter, was transformed to Agrobacterium tumefaciens strain AGL1. For oocyte expression the SbMATE2 coding region was cloned downstream of the T7 promoter in the USER compatible Xenopus expression vector pNB1u and linear template for in vitro transcription was generated by PCR. Further details can be found in the Supplementary information.

Transient expression and confocal microscopy. Transient expression in tobacco was performed by Agrobacterium infiltration of Nicotiana benthamiana leaves. Visualisation of the fluorescent protein fusions in epidermal cells or isolated mesophyll protoplasts was carried out using a Leica TCS SP5-II confocal microscope. Excitation/emission wavelengths were 515/525–535 nm for YFP and 435/500–510 nm for CFP. The excitation/emission wavelengths for capturing chlorophyll autofluorescence were 544/660–690 nm.

Oocyte transport assays. Oocytes from Xenopus laevis were obtained from EcoCyte Bioscience (Castrop-Rauxel, Germany). Capped cRNA of SbMATE2 was synthesized using the mMESSAGE mMACHINE® T7 Transcription Kit (ThermoFisher). For expression in oocytes, 25 μg of in vitro produced cRNA for the SbMATE2 transporter was injected into oocytes 4 days prior to performing transport assays essentially as described previously. Assuming an oocyte volume of ~1 μL, 50 nL of 2 mM compound stock solutions were injected to obtain estimated internal concentrations of 100 μM. Using the same needle each compound was injected into 25–30 oocytes expressing SbMATE2 and 25–30 control (non-expressing) oocytes. Following two washing steps, each batch of 25–30 oocytes was incubated for 90 min in 500 μL Kulori buffer at pH 5. After incubation, all intact oocytes were washed four times in ice-cold Kulori buffer pH 5 and 7–10 oocytes were extracted in triplicate in 50% MeOH as described previously. Extracts were analysed by LC-MS. Statistical significant differences between the means of SbMATE2 expressing and control oocytes were calculated using a t-test and GraphPad Software (www.graphpad.com).

Chemicals and LC-MS analysis. Amygdalin and cyanidin 3-O-glucoside were obtained from Sigma-Aldrich and indol-3-yl-methyl glucosinolate from Cfm Oskar Tropitzsch GmbH. Dhurrin, prunasin, epipherodendrin and epidermin were chemically synthesized. LC-MS analysis was performed using a Zorbax SB-C18 column on an Agilent 1100 Series LC coupled to a Bruker HCT-Ultra ion trap mass spectrometer as described previously. Compounds were localised in extracted ion chromatograms as sodium adduct ions: dhurrin (m/z 334), prunasin (m/z 318), amygdalin (m/z 480), epidermin (m/z 284), epipherodendrin (m/z 284), cyanidin 3-O-glucoside (m/z 449), indol-3-yl-methyl glucosinolate (m/z 493). Relative quantification was based on peak area using Bruker-DataAnalysis 4.0 (Bruker Daltonik).

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Author Contributions
B.D., H.H.N., B.L.M. and F.R. designed the study. B.D. performed the localisation studies and transport assays. H.H.N. assisted with transport assays, M.S.M. synthesized chemical compounds, and C.E.O. performed LC-MS. F.R. wrote the manuscript with contributions from the other authors.
Additional Information

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