Short title: Metabolite dynamics during sorghum germination

Corresponding author: Nanna Bjarnholt nnb@plen.ku.dk

Article title: Matrix-assisted laser desorption/ionization-mass spectrometry imaging of metabolites during sorghum germination

All author names and affiliations:
Lucia Montini1,2, Christoph Crocoll3, Roslyn M. Gleadow4, Mohammed Saddik Motawia1,2, Christian Janfelt5, Nanna Bjarnholt1,2

Affiliations:
1 VILLUM Research Center for Plant Plasticity, Department of Plant and Environmental Sciences, University of Copenhagen, Frederiksberg 1871, Denmark
2 Plant Biochemistry Laboratory, Department of Plant and Environmental Sciences, University of Copenhagen, Frederiksberg 1871, Denmark
3 DynaMo Center, Department of Plant and Environmental Sciences, University of Copenhagen, Frederiksberg 1871, Denmark
4 School of Biological Sciences, Monash University, Clayton, Victoria 3800, Australia
5 Department of Pharmacy, Faculty of Health and Medical Science, University of Copenhagen, Universitetsparken 2, 2100 Copenhagen, Denmark

One sentence summary: Matrix-assisted laser desorption/ionization-mass spectrometry imaging reveals that biosynthesis and recycling of the plant defence compound dhurrin are key events during sorghum grain germination.

List of author contributions:
N.B. and L.M. conceived the project and the research plan; L.M. performed the experiments; C.C. and C.J. provided technical assistance with respectively LC-3Q-MS and MALDI-MSI; R.M.G. provided plant material; M.S.M. synthesized authentic standards; N.B. and C.J. supervised the experimental work; L.M. and N.B. analysed the data and wrote the manuscript.; all authors have reviewed and approved the final manuscript.
Abstract

Dhurrin is the most abundant cyanogenic glucoside found in sorghum spp. (*Sorghum bicolor*) where it plays a key role in chemical defence by releasing toxic hydrogen cyanide upon tissue disruption. Besides this well-established function, there is strong evidence that dhurrin plays additional roles, e.g. as a transport and storage form of nitrogen, released via endogenous recycling pathways. However, knowledge about how, when and why dhurrin is endogenously metabolized is limited. We combined targeted metabolite profiling with matrix-assisted laser desorption/ionization (MALDI)-mass spectrometry imaging (MSI) to investigate accumulation of dhurrin, its recycling products and key general metabolites in four different sorghum lines during 72 h of grain imbibition, germination and early seedling development, as well as the spatial distribution of these metabolites in two of the lines. Little or no dhurrin or recycling products were present in the dry grain, but their *de novo* biosynthesis started immediately after water uptake. Dhurrin accumulation increased rapidly within the first 24 h in parallel with an increase in free amino acids, a key event in seed germination. The trajectories and final concentrations of dhurrin, the recycling products and free amino acids reached within the experimental period were dependent on genotype. MALDI-MSI demonstrated that dhurrin primarily accumulated in the germinating embryo, confirming its function in protecting the emerging tissue against herbivory. The dhurrin recycling products, however, were mainly located in the scutellum and/or pericarp/seed coat region, suggesting unknown key functions in germination.
Cyanogenic glucosides (CNglcs) are nitrogenous defence compounds that are widely distributed in the plant kingdom (Gleadow and Møller, 2014). They are of particular interest due to their presence in several crops, including the economically important cereals barley (Hordeum vulgare) (Nielsen et al., 2002), wheat (Triticum aestivum) (Erb et al., 1981; Jones, 1998) and sorghum (Kojima et al., 1979; Poulton, 1990), and because they often accumulate in high amounts in a wide range of plants (Kojima et al., 1979; Swain and Poulton, 1994; Crush et al., 1995; Gleadow and Woodrow, 2002; Busk and Møller, 2002; Forslund et al., 2003; Jørgensen et al., 2005; Sanchez-Perez et al., 2008; Gleadow et al., 2008). The CNglcs exert their function as defence compounds by releasing hydrogen cyanide (HCN), which is toxic to many living organism and potentially auto-toxic to the plant (Lechtenberg, 2011). The HCN release is caused by β-glycosidase catalysed hydrolysis of the CNglcs, initiated by tissue disruption, as for example caused by chewing herbivores (Morant et al., 2008). Besides this well-established function, CNglcs serve roles as storage and transport forms of reduced nitrogen to be re-incorporated in general metabolism upon demand (Selmar, 1993; Swain and Poulton, 1994; Pičmanová et al., 2015; Nielsen et al., 2016; Del Cueto et al., 2017; Bjarnholt et al., 2018; Schmidt et al., 2018). Although all plants possess a detoxification and nitrogen recovery pathway for HCN produced as a by-product of ethylene biosynthesis (Matilla, 2000; Piotrowski, 2008; Iqbal et al., 2013), nitrogen recovery from CNglcs proceeds via pathways that avoid release of the auto-toxic HCN. One such pathway has been elucidated (Bjarnholt et al., 2018), while the existence of others is documented by the coherent series of CNglc derived metabolites identified in several cyanogenic plants. These include CNglcs decorated with extra sugars or acyl groups, or amide and carboxylic acid derivatives (Li et al., 2013; Pičmanová et al., 2015; Blomstedt et al., 2016; Del Cueto et al., 2017; Bjarnholt et al., 2018; Schmidt et al., 2018b). A number of studies have indicated that CNglcs and possibly their metabolites are intimately involved in modulation of plant growth, development and stress responses (Jørgensen et al., 2005; Møller 2010; Blomstedt et al., 2012; Del Cueto et al., 2017; Ionescu et al., 2017).

Sorghum contains the tyrosine derived CNglc dhurrin and the dhurrin derived metabolites shown in Fig. 1 (Pičmanová et al., 2015; Blomstedt et al., 2016; Bjarnholt et al., 2018). The biosynthetic pathway and the enzymes catalysing dhurrin biosynthesis are well known (Gleadow and Møller, 2014), and the genes and enzymes forming a dhurrin recycling pathway have also been identified (Bjarnholt et al., 2018). This pathway proceeds via an intermediate glutathione conjugate (Fig. 1; 5) to form the end products ammonia and p-hydroxyphenylacetic acid. The latter compound exhibits hormonal activity in algae (Fries, 1976; Fries 1977) and it is stored as the glucoside p-glucosyloxyphenylacetic acid in sorghum (Fig. 1; 6) (Pičmanová et al., 2015; Blomstedt et al., 2016;
Bjarnholt et al., 2018). The dhurrin derived carboxylic acid p-hydroxymandelic acid has antifungal and allelopathic activity (Kope et al., 1991; Yamamoto et al., 1999; Gu et al., 2008) and is likewise stored as a glucoside (dhurrin acid) (Fig 1; 3) in high concentrations in sorghum (Pičmanová et al., 2015; Blomstedt et al., 2016). The sorghum grain contains little or no dhurrin (Tipples and Norris, 1983; Panasiuk and Bills, 1984; Veličković et al., 2014; Nielsen et al., 2016), but dhurrin is rapidly biosynthesized at the early stages of plant development where it accumulates in high amounts to protect the newly formed tissues (Halkier and Møller, 1989; Busk and Møller, 2002). Interestingly, dhurrin recycling occurs concurrently with dhurrin de novo biosynthesis at the early stage of sorghum seedling development (Adewusi, 1990; Busk and Møller, 2002; Bjarnholt et al. 2018). At that stage, nitrogen recovery may not be the main purpose of dhurrin recycling. Previous studies did not investigate if dhurrin biosynthesis and recycling takes place already during seed germination.

The seeds, formally called caryopsis, of Poaceae are characterized by a typical tissue compartmentalization, which depends on their taxonomical clade. Grains of cereals such as sorghum are constituted mainly by a starch and protein filled endosperm and a dormant embryo composed of the embryonic axis and scutellum (Dante et al., 2014) (Fig. 2). Seed germination and initiation of central metabolism is induced by imbibition and hormone controlled re-mobilization of resources from
the endosperm storage tissue (Yamaguchi, 2008; Sponsel, 2016). Hydrolytic enzymes produced in the aleurone layer surrounding the endosperm are released to degrade the storage polymers. The released general metabolites are then transported via the scutellum into the developing embryo as a source of energy and building blocks (Dante et al., 2014; Rosental et al., 2014) ensuring rapid growth of the vulnerable young seedling. Channelling of resources towards formation of specialized metabolites would thus entail that these play a role in germination or early growth of the seedling. In germinating barley seeds, hordatines have been found to accumulate in the embryonic axis, presumably for the purpose of defending the tissue against pests and diseases at this vulnerable growth stage (Gorzolka et al., 2016). Plant hormones and specialized metabolites are biosynthetically and structurally closely related, and several specialized metabolites are suspected of playing a role in hormonal signalling. The production of hydrolytic enzymes excreted by the aleurone and scutellum layers is triggered by gibberellins, hormones produced in the scutellum and transported to the aleurone cells (reviewed by Sponsel, 2016; Yamaguchi, 2008). In wheat for example, the phenolic specialised metabolite coumarin has been shown to induce production of hydrolytic enzymes in aleurone cells (Saleh and Abu El-Soud, 2015). Tissue-wise localization of compounds during early germination and seedling development may, therefore, lend clues to their significance and biological activity.
In this study we investigate the spatial distribution of dhurrin and its derivatives in the germinating sorghum grain using mass spectrometry imaging (MSI). This technique enables determination of the in situ localization of metabolites based on their molecular masses. A number of different methods are available, but the most popular is MALDI-MSI (Matrix Assisted Laser Desorption Ionization-MSI) (Bjarnholt et al., 2014; Boughton et al., 2016). This method allows the detection of a wide variety of metabolites and offers the possibility of very high spatial resolution (so far demonstrated down to 5 µm in plant tissue analyses) (Bjarnholt et al, 2014; Korte et al., 2015; Boughton et al., 2016). Coupling the MALDI ion source with mass analysers that provide high mass accuracy (< 2 ppm), such as the Orbitrap, provides identification of compounds based on their accurate mass. Here, we applied highly sensitive LC-triple quadrupole MS analyses in combination with MALDI-Orbitrap-MSI to study the emergence and spatial distribution of dhurrin and its derivatives compared with selected general metabolites. To gain more insight into the significance of dhurrin biosynthesis and its endogenous recycling pathways, we monitored the changes in accumulation of dhurrin and its derived metabolites during the early stages of the germination process, from the dry seed until the protrusion and early growth of the coleoptile where dhurrin biosynthesis is at its highest rate (Busk and Møller, 2002), in four varieties of sorghum, one of which lacked the capacity to synthesise dhurrin (Blomstedt et al., 2012).
Results

To explore the formation and recycling of dhurrin in the course of grain imbibition and germination, four different genotypes of *Sorghum bicolor* (L.) Moench were analysed. The wild types used were a commercial breeding line (wild type parent) (Blomstedt et al., 2012) and the cultivar BTx623 for which the genome sequence has been reported (phytozome.org). The mutant line *tcd1* (totally cyanide deficient) obtained by ethyl methanesulfonate mutagenesis (EMS), does not produce dhurrin because it carries a mutation in CYP79A1 rendering it inactive (Blomstedt et al., 2012). The *tcd1* line was generated in the commercial breeding line (Blomstedt et al., 2012) and has now been back-crossed twice into the BTx623 cultivar. This was included as a unique reference system to confirm the identities of dhurrin and its derivatives detected in the wild type, and to explore their metabolic relationship and possible significance in the germination process. A sibling from the last *tcd1*×BTx623 cross named TCD1, which does not contain the mutation, was analysed as an additional control.

Grains and seedlings from all four genotypes were collected at specific time points (Fig. 2A) following imbibition and germination in the dark. All samples were subjected to quantitative metabolite analysis by LC-MS. Selected BTx623 and *tcd1* samples were analysed by MALDI-MSI to monitor the spatio-temporal developments in accumulation of interesting analytes at four stages of germination (Fig. 2; B, C). The grains were imbibed for 12 h, and the levels of dhurrin and its recycling products were quantified in the whole-grain extracts at 0, 12, 24, 36, 48, 60 and 72 h after imbibition (AI), as well as in samples from the mature, un-imbibed grain.

Accumulation of dhurrin and its recycling products during germination and seedling formation

Analyses of the dry un-imbibed grain of the three dhurrin producing sorghum lines confirmed the previous observation from other genotypes that dhurrin is absent in the dry grain (Tipper and Norris, 1983; Panasiuk and Bills, 1984; Veličković et al., 2014; Nielsen et al., 2016). Previously, the earliest reported presence of dhurrin was in shoots after a total of 44 h of imbibition and germination in the dark (Halkier and Møller, 1989). In the current study of germinating grain and the germinated grain including emerging shoots and roots, dhurrin reached detectable levels already at 0 h AI in BTx623 and at 12 and 24 h AI, respectively, in the TCD1 and wild type lines (Fig. 3; A, B, C). Dhurrin levels were generally found to be higher in BTx623 compared to other dhurrin producing lines and continued to increase in BTx623 at the end of the experiment, whereas it was more stagnant in the two other lines. At the end of the experiment, at 72 h AI, the dhurrin concentration in BTx623 and TCD1 was approximately 1.3 nmol mg⁻¹ (SE = 0.08) and 1.1 nmol mg⁻¹ (SE = 0.04) respectively. In the *tcd1* parent line dhurrin content peaked at 48 h AI (0.9 nmol mg⁻¹, SE = 0.08).
The targeted analysis also documented the presence of previously reported dhurrin derivatives, namely the dhurrin glucosides (Fig.1; 4), dhurrin acid (Fig.1; 3), p-hydroxyphenyl(S-glutathione)acetonitrile (GS-pOHPACN) (Fig.1; 5) and p-glucosyloxyphenylacetic acid (pGPAAc) (Fig.1; 6). Other products and intermediates of the proposed recycling pathways were not detected (Fig. 1; 2, 7, 8). With a few specific exceptions discussed below, the accumulation patterns of the detected metabolites matched their expected formation from dhurrin, i.e. in most cases they were present in detectable levels at one or two time points after the appearance of dhurrin, and the concentrations were either increasing or levelling out towards the end of the experiment, similar to those of dhurrin.

An overall trend of proportionality between the level of dhurrin and the recycling products was observed, i.e. the tcd1 parent samples exhibited the lowest levels of dhurrin and also of recycling products compared to the other wild type lines. Average values and standard errors for all compounds/time points and lines are presented in the Supplemental Table S1. The dhurrin derivative present at the highest concentration was p-glucosyloxyphenylacetic acid (pGPAAc). The timing of the appearance of p-glucosyloxyphenylacetic acid was slightly shifted between the lines, and in the tcd1 parent line the concentration of p-glucosyloxyphenylacetic acid was already at its maximum level at the first time point where dhurrin was detected (Fig. 3C). After the initial increase in concentration all
dhurrin-producing lines maintained a stable level at 0.3 nmol mg\(^{-1}\) throughout the experimental period. The same accumulation pattern was observed for GS-pOHPACN, although the concentration of this compound was much lower reaching 0.006 nmol mg\(^{-1}\), 0.002 nmol mg\(^{-1}\) and 0.0011 nmol mg\(^{-1}\) in BTx623, TCD1 and \textit{tcd1} parent line, respectively (Supplemental Fig. S1). Such a pattern is in accordance with the position of GS-pOHPACN as an intermediate in the recycling pathway of dhurrin to \(p\)-glucosyloxyphenylacetic acid (Bjarnholt et al., 2018). In all three wild types, dhurrin acid was present as the second highest accumulated recycling product, reaching approximately 0.1 nmol mg\(^{-1}\), 0.06 nmol mg\(^{-1}\) and 0.03 nmol mg\(^{-1}\) in BTx623, TCD1 and parent, respectively. Although at barely detectable levels (between 0.005- 0.01 nmol mg\(^{-1}\)), dhurrin acid was also detected in dry mature seeds. Strikingly, the mutant line \textit{tcd1} accumulated \(p\)-glucosyloxyphenylacetic acid to roughly the same level as the wild type and sibling lines (Fig. 3D), but with a distinct time delay of approximately 24 h. This was unexpected, as \textit{tcd1} in the original parent background was previously reported to not accumulate \(p\)-glucosyloxyphenylacetic acid during plant development (from 7 to 46 d after sowing) (Blomstedt et al., 2016; Bjarnholt et al., 2018). In the present study, we did detect trace amounts of dhurrin in one replicate from each of two growth stages, indicating that it may in fact be produced in the mutant line at levels below the detection limit. However, at this stage it cannot be concluded whether dhurrin was actually present and could be a precursor for the detected \(p\)-glucosyloxyphenylacetic acid.

Accumulation of free amino acids

To investigate the timing of the onset of dhurrin biosynthesis in more detail, we compared it with one of the very early events in general metabolism during seed germination: the increase in concentration of free amino acids, caused by hydrolysis of storage proteins or \textit{de novo} biosynthesis, to feed the need of the developing embryo. Dhurrin is biosynthesized from the amino acid tyrosine, and furthermore, the recycling of dhurrin into dhurrin acid and \(p\)-glucosyloxyphenylacetic acid leads to release of ammonia which is toxic in high concentrations and therefore efficiently assimilated into amino acid metabolism via incorporation into glutamate and glutamine. It can therefore be envisioned that the ratio or concentration of some or all free amino acids may be affected in the mutant line compared to the dhurrin producing control lines. Fig. 4 displays the development in the total sum of free amino acids in the four different lines. It should be noted that glycine could not be detected with the applied method. However, its concentration is expected to be relatively small (Feenstra et al., 2017). Differences were observed in the concentrations between the individual amino acids (Supplemental Fig. S3), but the overall concentration trajectories over time as well as between the sorghum lines were roughly identical to that displayed for the total sum. The concentrations of individual free amino acids in the dry grain varied from below 0.01 nmol mg\(^{-1}\) for tryptophan to around 1 nmol mg\(^{-1}\) for asparagine. In all lines, the concentrations of free amino acids increased immediately upon imbibition,
but initially at a slow rate. A dramatic increase was observed at 24-48 h AI, with roughly the same
differences between the wild type control lines as was observed for dhurrin accumulation. As
exemplified by the plot of the total free amino acid content in Fig. 4, the levels were generally highest
in BTx623 and consistently lower in the parent line. The sibling TCD1 line was quite similar to
BTx623 until 48 h AI. After 48 h, the concentrations of free amino acids in the parent and TCD1 lines
levelled out or even decreased, while they were still increasing in the BTx623 line. These trajectories
and line-dependent differences completely matched the dhurrin trajectories (Fig. 3). Differences in
concentrations of free amino acids in germinating seeds were previously shown to depend on the
 genetic background (Feenstra et al., 2017). As previously mentioned, the tcdl line was originally
generated in the genetic background of a near isogenic commercial breeding line, and the tcdl and
TCD1 lines used in the present study are the F2 generation of the tcdl×BTx623 cross. Consequently,
these two lines have a mixed parent×BTx623 genetic background which is presumably what is
reflected by the somewhat intermediate position of TCD1 between BTx623 and the mutant parent line
with regards to concentrations of dhurrin and free amino acids. Interestingly, the free amino acid
levels were different in tcdl compared to its sibling TCD1, as illustrated for the total sum in Fig. 4.
The tcdl line seemed to be delayed with respect to amino acids accumulation until approximately 48 h
AI, after which the levels were often higher in tcdl compared to TCD1 and consequently also the

Figure 4. Total free amino acids content during germination and early seedling development in four sorghum lines. The bars show the total sum of all free proteinaceous amino acids, except glycine which could not be quantified with the applied method but is usually present in low amounts. Data for individual amino acids are shown in Fig. S5. Values are mean of n = 3 replicates for each time point. The error bars represent the standard error within the biological replicates.
parent line. With only three biological replicates to measure changes in such a dynamic pool of metabolites undergoing rapid change, the SEs were relatively high. However, glutamine, proline and tyrosine stood out, as the apparent levels of these amino acids reached higher or similar levels in \textit{tcd1} than in BTx623 at one or more of the time points 48-72 h AI (Supplemental Fig. S3). Almost all other amino acids were higher in BTx623 than in all other lines at most time points, with the most pronounced differences being found for aspartate, asparagine, histidine, isoleucine, methionine, serine, threonine and valine. Levels of alanine, arginine and lysine appeared somewhat unaffected or differently affected by the genetic background, and the remaining amino acids followed the trends of the total sum, but with moderate differences between the lines (Supplemental Fig. S3).

\textbf{Spatial distribution of general and specialized metabolites}

The stages selected for MSI analyses were chosen by a combination of the metabolite analyses and development of particular anatomical features. The four stages were: \textit{S}_0, the dry grain representing a base line, \textit{S}_1, the imbibed grain which was the first time point where dhurrin was detected in the wild type, \textit{S}_2, where the radicle and early shoot emerged and dhurrin recycling products were first detected, and \textit{S}_3, the stage where the primary root was fully developed and the coleoptile, containing the primary leaf, completely protruded from the embryonic axis of the embryo. For BTx623 these stages corresponded to the time points shown in Fig. 2. The development of \textit{tcd1} was slightly delayed compared to BTx623 and therefore the seeds selected for MSI analysis from these lines were chosen based on the developmental stage rather than time (time points shown in Fig. 3D). After the first 36 h AI the mutant line was delayed by approximately 12 h compared to the wild type. At this stage the radicle and the coleoptile containing the primary leaves were fully emerged in BTx623, whereas in \textit{tcd1} that was delayed until 48 h AI. For each stage and line, three seeds (including roots and seedlings when present) were sectioned longitudinally at 10 µm thickness, placed on pre-cooled glass slides and immediately freeze-dried, using the method reported by Kawamoto (2014), with the few modifications reported by Gorzolka et al. (2016), Boughton and Thinagara (2018), Sarabia et al. (2018) and Schmidt et al. (2018). Three consecutive sections were selected and subjected to respectively fluorescence microscopy and MSI analyses in both positive and negative mode to allow detection of an extensive range of metabolites.

Dhurrin and its recycling products and a number of general metabolites were detected in positive mode using 2,5-dihydroxybenzoic acid (DHB) as matrix. Amino acids were detected in negative mode using 1,5-diaminonaphthalene (DAN) as matrix on a different section. To minimize the crystal size and obtain uniform matrix deposition and thereby minimize delocalization of analytes, sublimation
was used for matrix deposition (Hankin et al. 2007). The sections were analysed with 30 µm pixel size which provided sufficient spatial resolution for matching the morphological features observed in the fluorescence microscopy images while maintaining a reasonable acquisition time (~ 4-6 h per sample). During data processing to produce the images, each ion was normalized to the total ion count in each pixel, making up for some of the variation between analyses. Many factors, including sample handling, matrix deposition and plant-tissue background can cause differences in ionization efficiencies for individual metabolites across the sample. Potentially this may result in images of metabolites distribution that do not provide a true representation of the sample constituents. To validate our results, we therefore compared with previous data obtained using different approaches.

During the germination process, breakdown of the starchy endosperm into mono- and disaccharides provides an important source of energy for the developing embryo (Lopes and Larkins, 1993; Aoki et al., 2006; Leonova et al., 2010; Sánchez-Linares et al., 2012; Yan et al., 2014; Feenstra et al., 2017). Quantification of mono- and disaccharides of micro-dissected germinating cereal seeds from maize (Zea mays) and oat (Avena sativa) showed that mono-hexose (Hex) and dihexose (Hex2) accumulate in the embryo and scutellum (Leonova et al., 2010; Feenstra et al., 2017). The same distribution was observed in the MALDI-MSI analyses of the germinating sorghum seeds (Fig. 5A). Hex2 was lowly abundant in un-imbibed samples, but its signal greatly increased in the embryo and scutellum already after imbibition (Si). As the germination proceeded and the axis protruded, Hex2 was concentrated more extensively in the emerging radicle and coleoptile (S2) (Fig. 5A). Accumulation of Hex occurred after the protrusion of the radicle from the embryo (S1), where it was mainly found in the differentiation/elongation zone of the emerging radicle. At the last stage analysed (S3), the Hex signal was highly concentrated in the coleoptile and slightly less abundant in the radicle. At the S0 stage, Hex and Hex2 were detected in the pericarp/aleurone layers (the two layers could not be distinguished with the lateral resolution applied in the MSI analyses). This is consistent with observation of MALDI-MSI in germinating maize kernels (Feenstra et al., 2017).

It is important to note that the compounds are detected based on their accurate mass and corresponding sum formulae. In the scutellum and developing embryo Hex and Hex2 are the major general metabolites glucose and sucrose, as demonstrated in the microdissection experiments (Leonova et al., 2010; Feenstra et al., 2017), however it is possible that Hex and Hex2 in the layer(s) encasing the seeds are different saccharides. For Hex and Hex2 the potassium adducts \([C_6H_{12}O_6+K]^+\) and \([C_{12}H_{22}O_{11}+K]^+\) gave the strongest signals in positive mode and were therefore used to map the distribution of these compounds. This was in accordance with the maize kernel study using a similar MALDI-MSI method (Feenstra et al., 2017) which has also been shown to provide a good signal quality from Hex2 in...
based on this knowledge the efficiency and consistency of our ionization methods were compared by analysing the distribution of Hex and Hex$_3$ as deprotonated species ([$C_6H_{12}O_6$H]⁻ and [$C_{12}H_{22}O_6$2H]⁻) detected in negative mode (Fig. 5A). At all stages analysed, the distribution of [Hex+K]$^+$ and [Hex$_3$+K]$^+$ vs. [Hex-H]$^-$ and [Hex$_3$-H]$^-$ were as identical as can be expected for different sections, demonstrating the efficiency of both ionization methods.

Choline is an important plant constituent as a precursor in the formation of many metabolites including acetylcholine and phosphatidylcholine (PC) (Miura et al., 1984). In chick pea (Cicer arietinum) seedlings, choline is highly abundant in the cotyledon and growing part of the embryo together with many other seed oil reserves (Fincher, 1989; Leonova et al., 2010). The same tissue-specific distributions were demonstrated for germinating barley seeds using MALDI analysis, showing strong accumulation of PC in the endosperm whereas choline was detected in the scutellum+embryo and the aleurone layer (Gorzolka et al. 2016). Similar to Gorzolka et al. (2016) we detected choline as protonated species and PC (16:0) as a potassium adduct (respectively [$C_4H_{13}NO_1$+H]$^+$ and [$C_{24}H_{50}NO_7$+K]$^+$) in positive mode. These analyses demonstrated that our MALDI-MSI method...
afforded efficient ionization across all examined germination stages and tissues, including the endosperm (Fig. 5B). Nearly identical results were obtained using the tcd1 mutant line (Supplemental Fig. S5), demonstrating similar ionization efficiencies and basic metabolite distributions in the two genotypes and corroborating the robustness of the methods.

Localization of dhurrin and its recycling products during germination and seedling formation

Dhurrin and its recycling products were detected as potassium or sodium adducts in positive mode. The ions providing the most intense signal were selected for generation of the images. Dhurrin was most consistently detected as a potassium adduct ([C_{14}H_{17}NO_{7}+K]^{+}), and only in a few individual replicates also detected as its sodium adduct with much lower signal intensity. Therefore, the potassium adduct was used to generate the images. Dhurrin glucosides were the only recycling products detected as a potassium adduct [M+K]^{+}, whereas dhurrin acid, GS-pOHPACN and pGPAAc were exclusively detected as their sodium adducts ([M+Na]^{+}). Supplemental Table S3 provides a list of accurate masses applied to generate the images. The intensities varied between replicates, but the distributions were the same. The most clear results were selected for presentation in Fig. 6, and the replicate results can be viewed in Supplemental Fig. S7. In the metabolic extract analysis, dhurrin was first detected after imbibition (S_i) in the BTx623 line, and the recycling products were first detected after axis protrusion (S_1). In the MSI analyses these compounds were all detected in the dry seeds as well as after imbibition (Fig. 3 + Fig. S7, S_0 + S_i), but the signal intensities were minute at these early stages, and the compounds not detected in all replicates. This explains why the very same constituents were not detected in the total extracts that were analysed by LC-MS, due to the dilution effect. The weak signals make it difficult to assess the exact localization of the compounds, but it appears that dhurrin and its glucosides were present in both scutellum and embryo of the dry seeds. The three dhurrin glucosides have the same sum formula and thus identical exact mass and could therefore not be differentiated in the MSI analysis. The distribution of the ions corresponding to the dhurrin glucosides consistently followed the same distribution pattern as dhurrin until the end of the experiment. On the other hand, the dhurrin recycling products dhurrin acid and p-glucosyloxyphenylacetic acid mainly accumulated in the scutellum and other tissues enclosing the embryo. After water uptake, this difference was much more pronounced than at S_0, as the signal for dhurrin and the dhurrin glucosides were clearly much more intense in the embryo than in the scutellum while the recycling products seemed to be almost exclusively located in the scutellum (Fig. 6, S_i). As the germination proceeds, the radicle is the first part of the seedling to visibly emerge from the seed coat. Subsequently, the coleoptile containing the plumule (primordial leaf) protrudes from the embryonic axis, and this marks the end of germination. In BTx623, this developmental stage, S_1, was
reached at 24 h AI. At this stage the dhurrin and dhurrin glucosides appeared to be more concentrated in the differentiation/elongation zone of the emerging radicle and in the area protecting the plumule. The remaining compounds were still mainly found in the scutellum.

At the last time point, S2, all compounds were exclusively detected in the newly formed root and coleoptile tissue. In particular, dhurrin was detected at very high intensity in the tip of the fully emerged coleoptile, consistent with a previous study (Halkier and Møller, 1989). The dhurrin derivatives were weakly detected at this stage and there were variations between replicates (Supplemental Fig. S7), making it difficult to determine whether the compounds were uniformly distributed or not. The strongest signal was found for the dhurrin glucosides, while dhurrin acid, p-glucosyloxyphenylacetic acid and GS-p-OHPACN were barely detectable. The distribution of GS-pOHPACN diverged from the remaining compounds, as it was generally more broadly distributed. At S0 it was detected at highest intensity in the embryo, whereas at S1 it was equally intense in embryo and scutellum. In addition, in some replicates it was detected in the pericarp/aleurone layer at S0, S1 and possibly S2. As such, GS-pOHPACN and p-glucosyloxyphenylacetic acid, that are respectively
intermediate and product of the same metabolic pathway, displayed only partially overlapping
distributions.

At all imaged stages, MSI signals corresponding to dhurrin and its recycling products were absent in
the \textit{tcd1} mutant, at the applied mass tolerances (Table S3) and default abundance threshold of
MSiReader. This is visualized for dhurrin in Fig. S6A, showing average mass spectra of BTx623 and
\textit{tcd1} at S1. This is difficult to visualize for earlier stages, where dhurrin and its derivatives were only
detected in scattered pixels in BTx623 samples, making the average spectra uninformative. For
dhurrin the most abundant isotope of the [M+K]⁺ ion is that containing a single ¹³C which has a
theoretical abundance of 15.1 %. In the three pixels with highest dhurrin abundance in the S₀ sample
shown in Fig. S7, we were able to detect this isotope (m/z 351.0670) at respectively 8 %, 9 % and 15
% of the monoisotopic [dhurrin+K]⁺ peak (Fig. S6C). Given the very low absolute peak intensities,
which affects the accuracy of the measured ratios, and that this m/z value was absent from other pixels
and \textit{tcd1} samples, this further supports the presence of low quantities of dhurrin and its derivatives in
the BTx623 line at S₀ and S₁. The second most abundant isotope is [dhurrin⁺⁴¹K]⁺, however at a
theoretical abundance of 7.2 %, this m/z value was not detectable at the S₀ and S₁ stages. At S₁ and S₂,
the MSI findings were corroborated by the LC-MS based metabolite analyses of the \textit{tcd1} mutant (Fig.
3D). Taken together, the results support that the detected ions in BTx623 do in fact correspond to the
proposed compounds. \textit{p}-Glucosyloxyphenylacetic acid was detected at later time points in the LC-MS
analyses of \textit{tcd1}, but unfortunately the size of the protruded axis (root and coleoptile) at those stages of
seedling growth caused embedding and sectioning to be impossible and therefore the distribution of
this compound could not be explored by MSI.
In the germinating maize seeds only three proteinaceous amino acids were reliably detected by MALDI-MSI (Feenstra et al., 2017). In our study, all amino acids except alanine and glycine were readily detected as deprotonated species in negative mode [M-H]. However, the relative signal intensities in the MS images did not completely reflect the concentrations determined in the LC-MS analyses. This serves to emphasize the well-known fact that the ionization efficiency in MS imaging depends on both the type of applied matrix, the tissue matrix background and in particular the physicochemical properties of the analytes. Fig. 7 shows results for a selected subset of amino acids with a full data set provided in the supplementary data (Supplemental Fig. S8 and Fig. S9). Although MSI is not a quantitative technique, the overall trends in intensity of amino acid ions in images from samples of increasing age did match the overall trends found in the analysis of free amino acids in extracts. In BTx623 as well as in tcd1, there was a sharp increase in signal intensity from S1 to S2 for most amino acid ions, reflecting the sharp increase in amino acid concentrations detected in extracts of whole samples at 24-36 h AI. Aspartate, glutamate and asparagine were present in high concentrations at all stages and genotypes analysed by LC-MS and these amino acids were also detected at all stages in the MSI analyses of BTx623 and tcd1 (Fig. 7; Supplemental Fig. S8 and Fig. S9). In general, small, neutral amino acids such as valine, methionine, leucine/isoleucine (which have identical sum formulae and cannot be differentiated in the MSI analyses), serine, proline and threonine were only clearly detectable by MSI at the later growth stages, regardless of their concentration in the extract analysis. Conversely, lysine and tryptophan were found in low concentrations at all growth stages, but were nevertheless in some cases detectable in the MSI analysis already at stage S1.

No free amino acids were detected in the endosperm, indicating that the rate of hydrolytic release of free amino acids from the storage proteins was lower than the transport of the amino acids towards the developing tissues. At the active growth stages from S1 onwards, where the amino acids are also synthesized *de novo*, the presence of amino acids was detected both in the scutellum and embryo. At these early stages of germination, the free amino acids are required for biosynthesis of protein and metabolites, presumably in both tissues, but eventually only the embryo will be active. This was reflected by the MSI results with a tendency towards the scutellar amino acid signals decreasing and the embryonic axis localized amino acid signals increasing from S1 to S2. Aspartate, glutamate, glutamine and tryptophan were in some cases also detected in the aleurone/pericarp layer at stage S0 and/or S1. The inconsistency in detection between stages (Supplemental Fig. S8) and replicates (Supplemental Fig. S9) may be exacerbated by the low concentrations present. At the last imaged growth stage, some amino acids displayed distinct localizations in the newly formed tissues. For
example, the highly abundant glutamic acid was almost detected everywhere, whereas lysine was
mainly detected in the elongation zones of both root and shoot where tryptophan on the other hand, was specifically not detected (Fig. 7). We observed no striking differences for \textit{tcd1} compared to BTx623, as illustrated for a subset of amino acids in Fig. 7B and Supplemental Fig. S9.
Discussion

The spatio-temporal investigation of the accumulation of dhurrin and its recycling products in the germinating sorghum grain demonstrates that initiation of dhurrin biosynthesis and even the endogenous metabolism of dhurrin are very early events in seed germination and seedling development, initiated already during imbibition. The *in situ* localization of dhurrin metabolites showed that dhurrin and related cyanogenic di-glucosides accumulate in the newly formed tissues, protecting the vulnerable developing embryo. On the other hand, dhurrin derivatives accumulate mainly in the scutellum, or are distributed across embryo, scutellum and aleurone/pericarp layer, indicating key functions in the germination process.

Imbibition of the dry seeds is the starting point of germination. In the model plant Arabidopsis (*Arabidopsis thalian*) metabolite abundances undergo substantial changes after the first few hours of imbibition (Rajjou and Debeaujon, 2008; Howell et al., 2009). The metabolic activity generates the essential building blocks for plant growth, energy production and protein biosynthesis. In accordance with previous studies we found only low concentrations of free amino acids in the dry sorghum grain (Bewley et al., 2013; Feenstra et al., 2017). The concentration of total and most of the individual free amino acids increased slowly at the first few time points after imbibition, and then increased dramatically at 24 h AI in BTx623 and slightly later or less steeply in the other control lines (Fig. 4; Supplemental Fig. S3). The trajectories of dhurrin concentration were exactly the same (Fig. 3), showing that dhurrin biosynthesis was switched on as early as the key event of amino acids release from the endosperm storage proteins. This suggests that dhurrin is of primary importance in the early phase of germination. Dhurrin localization at the tissue level as determined by MALDI-MSI, furthermore points towards the presumed primary functionality of the metabolite. The presence of dhurrin in the differentiation/elongation zone of the emerging radicle and in the area protecting the plumule confirms the position of dhurrin as a key player in the sorghum defence system. Specialised metabolites that provide defence against general biotic stresses are commonly found in peripheral or key tissues where they can provide most benefit to the plant as a first line of chemical defence (Bellotti and Riis, 1994; Ha et al., 2012; Shroff et al., 2008; Schmidt et al., 2018b). In germinating barley, the antifungal hordatines were found to accumulate predominantly in the outer layer of the shoot (Gorzolka et al., 2016), similar to dhurrin accumulation in the outer layer of the cotyledon. However, the concurrent onset of the dhurrin recycling processes early in the development is indicative of other roles in the germinating grain.
The dhurrin glucosides were detected in the metabolite extracts as soon as dhurrin levels increased at 24 h AI. In the MS images of BTx623, they were co-localized with dhurrin. The biological functions of these di-glucosides are unknown, but as their aglycone is the dhurrin cyanohydrin, they will release HCN following possible hydrolysis resulting in the removal of both glucose residues. The cyanogenic di-glucosides found in cassava (*Manihot esculenta*) and the rubber plant (*Hevea brasiliensis*), have been proposed to be transport forms protected against cleavage by the mono-glucosidase that hydrolyses the cyanogenic mono-glucosides (Selmar, 1993). By contrast, in almonds (*Prunus dulcis*, syn. *Prunus amygdalis*), the cyanogenic di-glucoside amygdalin accumulates in the kernels and is responsible for the toxic cyanide release in the bitter seeds following the serial action of two glucosidases (Sanchez-Perez et al., 2008; Sánchez-Pérez et al., 2009; Sánchez-Pérez et al., 2012; Del Cueto et al., 2017).

Dhurrin-6′-glucoside was originally isolated from guttation droplets of sorghum leaves (Selmar et al., 1996) indicating a role in transport for this compound. The dhurrin glucosides may serve different functions in different tissues, e.g. transport forms in aerial tissues and defence purposes in roots where dhurrin is lowly abundant or absent. Determination of the distribution of the individual di-glucosides would require dissection and extract analysis where the isobaric compounds can be differentiated by retention time and their linkage types be determined by ion mobility MS (Pičmanová et al., 2015) or by NMR spectrometry of the isolated compounds.

The remaining dhurrin recycling products detected in the extracts of germinating seeds from the dhurrin producing lines either emerged at the same time as the dhurrin concentration increased (*p*-glucosyloxyphenylacetic acid) or shortly after (GS-*p*OHPACN), or were detected at all time points, but found to increase shortly after the increase in dhurrin concentration (dhurrin acid). This early emergence or increase likewise indicate important roles of these compounds at the early stages of plant life. In fact, all derivatives were detected already at S1 in the MS images, supporting their importance for the germinating seed or early seedling formation. Despite their apparent metabolic relation to dhurrin, the two major derivatives, dhurrin acid and *p*-glucosyloxyphenylacetic acid, were apparently absent from the growing tissues until after germination at S2. This would suggest that before this stage, supply of reduced nitrogen to the growing tissues is not the main purpose of the pathways for conversion of dhurrin into these acids. The presence of dhurrin acid in the dry seed and its localization around the embryonic axis after imbibition at S1 supports that it functions as a storage form of an antifungal agent (Kope et al., 1991), protecting the seed and the early embryo stage. In cassava, the main cyanogenic glucoside is linamarin, and its dhurrin acid analogue, linamarin acid, was found to be localized to the stem and in the vascular tissue of the cassava tuber (Schmidt et al.,...
2018b). The presence in the vascular tissue proposes that the glucosylated CNglc-derived carboxylic acids may be transport forms of their potentially bio-active aglucones.

*p*-Hydroxyphenylacetic acid, the aglucone of *p*-glucosyloxyphenylacetic acid has been shown to exert growth promoting effects and auxin-like activities in algae (Fries, 1976; Fries, 1977). The compound has been isolated from plants, algae and associated microbes, and its non-hydroxylated analogue phenylacetic acid, is well-established as an auxin-like plant hormone (Koepfli et al., 1938; Ferro et al., 2010; Simon and Petrášek, 2011). Glucosylation is a well-known mechanism used to control auxin homeostasis (Korasick et al., 2013) and the activity of gibberellins, both key hormones controlling seed germination (Yamaguchi, 2008). The observed localization of *p*-glucosyloxyphenylacetic acid in the scutellum, the tissue that is considered the main source of gibberellin production in germinating seeds, renders it tempting to suggest that *p*-hydroxyphenylacetic acid has an equally important role in sorghum seed germination. Assignment of an important function to *p*-hydroxyphenylacetic acid is supported by the fact that the *tcd1* mutant was shown to produce *p*-glucosyloxyphenylacetic acid, which after germination accumulated at wild type levels (Fig. 3D). The presence of the two dhurrin derived acids, dhurrin acid and *p*-glucosyloxyphenylacetic acid in *tcd1* was surprising in the light of previous reports where *p*-glucosyloxyphenylacetic acid was found to be absent or present in trace amounts in vegetative tissues of *tcd1* (Bjarnholt et al., 2018) and a second mutated line, *tcd2*, which is mutated in the last enzyme of dhurrin biosynthesis (Blomstedt et al., 2016). In the current study, we did detect traces of dhurrin in a few replicates of *tcd1* extracts, and this may be what fuels the production of the two acids in the germinating seeds and/or young seedling tissues of this line, with the low dhurrin supply explaining the delayed accumulation. On the other hand, the eventual accumulation of wildtype levels of *p*-glucosyloxyphenylacetic acid suggests that this compound is produced from a completely different pathway which remains to be elucidated.

GS-*p*OHPACN is an intermediate in the production of *p*-glucosyloxyphenylacetic acid from dhurrin (Bjarnholt et al., 2018). As such, it was convincing to find it localized to both embryo and scutellum, where its precursor, dhurrin, and its downstream product, *p*-glucosyloxyphenylacetic acid, accumulated. At S1, the accumulation of precursor and product appeared almost completely separated between the two tissues. The presence of GS-*p*OHPACN in both tissues could therefore suggest that it is produced from dhurrin in the embryo and subsequently transported to the scutellum for further processing into *p*-glucosyloxyphenylacetic acid. Compartmentalization between the different tissues of germinating seeds has also been suggested for the gibberellin biosynthetic pathway in Arabidopsis (Urbanova and Leubner-Metzger, 2016). However, this does not explain the presence of GS-*p*OHPACN in the aleurone/pericarp layer. In the aleurone tissue, the small molecules present can be expected to be mainly signalling compounds or products resulting from the cell wall degradation that
happens during germination. It can be hypothesized that GS-pOHPACN is a transport molecule of the bio-active non-glucosylated \( p \)-hydroxyphenylacetic acid or even of free pOHPACN (\( p \)-hydroxyphenylacetonitrile) which may have an undescribed bio-activity. \( p \)-Hydroxyphenylacetic acid was not detected in the extract analyses, maybe because the locally produced and aleurone localized \( p \)-hydroxyphenylacetic acid were diluted too much in the total extracts. Conversion of GS-pOHPACN to \( p \)-hydroxyphenylacetic acid requires the action of one of the two lambda GSTs (glutathione transferases) \( SbGSTL1 \) or \( SbGSTL2 \), followed by the nitrilase heterodimer \( SbNIT4A/B2 \). The transcripts of these enzymes co-localized at the cellular level in sorghum seedling leaves (Bjarnholt et al., 2018) and were co-expressed in developing sorghum seeds (Nielsen et al., 2016). Transcriptome analyses of the individual tissues in the germinating sorghum seed, such as recently carried out for barley (Betts et al., 2017), could help identify source and sink tissues of GS-pOHPACN and lend further clues as to the nature of its biological function.

The overall pattern of free amino acid accumulation showed that the siblings \( tcd1 \) and TCD1 were somewhat intermediate between their parent wild type lines. The \( tcd1 \) mutant displayed a delay in accumulation of free amino acids compared to its sibling. This delay was mirrored by the delay in germination in \( tcd1 \), as germination of TCD1 and the parent line were not significantly different from BTx623. Amino acids have been shown to influence the germination process. In Arabidopsis, \textit{de novo} biosynthesis of methionine was described to be of primary importance for seed germination and seedling growth (Gallardo et al., 2002). In other plants, such as barley and sugar beet, enzymes related to methionine metabolic pathways dramatically increase in the first hours after imbibition prior to radical emergence (Soeda et al., 2005; Catusse et al., 2008). In BTx623 and TCD1, the concentration of methionine increased much more steeply than in \( tcd1 \), in which comparable levels were reached with a delay of 24-36 h. It is possible that an imbalance in the free amino acids caused by possible over accumulation of tyrosine in the absence of the dhurrin biosynthesis, could influence the germination rate. Indeed, tyrosine as well as proline and glutamine accumulated to higher levels in \( tcd1 \) compared to its sibling and even BTx623 after germination. Like other aromatic amino acids, tyrosine is produced in the plastids and exported into the cytosol as precursor for synthesis of other metabolites. Degradation of tyrosine via a pathway initiated by tyrosine amino transferase (Tyr-AT) and transamination of \( \alpha \)-ketoglutarate into glutamate (Hildebrandt et al., 2015; Schenck and Maeda, 2018) avoids the risk of over-accumulation. Glutamate did not accumulate in the \( tcd1 \) mutant relative to the wild type, but as glutamate is a precursor for proline and glutamine, it is likely that the observed increase in levels of the latter two in \( tcd1 \) is a consequence of tyrosine over-accumulation and degradation. Tyrosine may also be directed into synthesis of other metabolites such as tyramine, tocopherols, Vitamin E and numerous phenolic compounds such as the flavonoids. We did not target
these compounds in our analyses, but it is also possible that an imbalance in accumulation of such metabolites may have caused the perturbation of the germination rate.

Our investigations of the MALDI-MSI method showed that the applied matrices worked similarly and well across tissue types, genotypes and growth stages for a range of general metabolites with different physicochemical properties (Fig. 5). Our MSI results for hexoses and amino acids were in accordance with other studies using both MALDI-MSI and LC-MS analyses of micro-dissected tissues (Fig. 5; Fig. 7) (Leonova et al., 2010; Gorzolka et al., 2016; Feenstra et al., 2017). Although MSI is not a quantitative technique, the overall trends in intensity of the amino acid ions in samples taken at different stages of the germination process (Fig. 4) matched the overall trends found in the analyses of extracts (Supplemental Fig. S3). Amino acids are poorly ionizable compounds and because of their amphiprotic nature they have been reported to be hardly detectable by MALDI-MSI (Toue et al., 2014; Feenstra et al., 2017). Despite this, we visualised the distribution of 18 out of the 20 free proteinaceous amino acids, many with highly intense signals. The very high selectivity of the Orbitrap operating at 140,000 mass resolving power (applying a bin width of 0.01 Th in image generation) has likely improved detection compared to experiments on low-resolution MALDI-TOF systems (Toue et al., 2014), but this does not explain the difference between the current study and the work of Feenstra et al. (2017). Factors such as sample preparation, mounting technique (CMC vs. gelatine), matrix (DAN vs. DHB), and sample drying (freeze drying vs. desiccation) as well as differences in plant background matrices might have a significant impact on signal intensity and therefore on the successful detection of metabolites of interest. Some discrepancies were indeed observed between the LC-MS and the MALDI-MSI analyses of dhurrin and dhurrin-derived metabolites. At the early stages of germination, the discrepancies might be explained by the more localized accumulation, allowing compounds to be more easily detected by MSI than in the total seed extracts. However, at the last point of seedling development investigated, where the radicle has evolved into the primary root and the coleoptile was fully emerged from the seed coat, the concentrations of dhurrin and its recycling products in the dhurrin producing wild type lines were just as high as at the previous growth stage, but only weakly or barely detected by MALDI-MSI. This may be explained by the substantial change in the plant background during the rapid transition from the seed to a seedling, which may have affected the ionization efficiency of the compounds. Ion suppression caused by matrix background is a common problem in MS-based studies.

This study demonstrates the power of combining LC/MS and MALDI-MSI to gain insight into important biological processes. The results have demonstrated an apparent importance of dhurrin and its recycling processes at the earliest stages of sorghum grain germination and seedling formation and
unveiled the high complexity of this metabolic grid and compartmentalisation. These insights point
towards specific physiological functions of dhurrin and its recycling products.
Materials and methods

Plant material

Seeds of four different sorghum (Sorghum bicolor) lines, namely BTx623, TCD1, tcd1 parent and tcd1 mutant, were imbibed for 12 h in the dark at room temperature and germinated on moist filter paper in petri dishes at 28 °C for 3 d. Samples were collected every 12 h at eight time points, dry grain and 0, 12, 24, 36, 48, 60 and 72 h, with 0 h corresponding to those taken immediately after 12 h of imbibition. At each time point, three seeds combined as one biological replicate (for a total of nine seeds for each time point) were immediately quenched in liquid nitrogen (N2), and used for metabolite quantification by LC-MS. For in situ visualization of metabolites in BTx623 and tcd1, seeds were collected at four stages based on the development of particular anatomical features, taking into account the different germination rates observed between the lines. To cover biological variation between individual seeds and technical variation between analyses, each time point was analysed by MSI in triplicates for BTx623 and duplicates for tcd1.

Sample preparation for metabolite profiling by LC-MS

Frozen plant material was ground in liquid nitrogen using mortar and pestle and extracted in 85 % v/v aqueous methanol with 0.5 % v/v formic acid (80 °C, 5 min) in screw-lid microtubes (Sarstedt, Germany) and immediately cooled on ice. The extracts were transferred to screw cap glass vials and stored at -20 °C until further pre-treatment. For analyses, samples were diluted 5 times (1:5 v/v) in water and filtered through a membrane filter (0.45 μm, Merck Millipore) by centrifugation (2000 g, 5 min). After filtration, samples were stored at 4 °C until analysis.

Quantification of dhurrin and related metabolites by LC-3Q-MS

The diluted extracts were directly analysed by LC-MS. Chromatography was performed on an Advance UHPLC system (Bruker, Bremen, Germany). Separation was achieved on a Zorbax Eclipse XDB-C18 column (100 x 3.0 mm, 1.8 μm, 100 Å, Agilent Technologies, Santa Clara, CA, USA), using a gradient of A = MilliQ grade water with 0.05 % v/v formic acid, and B =100 % methanol. The elution profile was: 0-0.03 min, 2 % B; 0.03-0.9 min, 2-15 % B; 0.9-1.4 min. 15-60 % B; 1.4-3.3 min, 60-100 % B; 3.3-3.9 min, 100 %; 3.9-4.0 min, 100-2 % B; and 4.5-5.0 min 2 % B. The mobile phase flow rate was 500 μl*min⁻¹ and column temperature was maintained at 40 °C. The liquid chromatography was coupled to an EVOQ Elite TripleQuad mass spectrometer (Bruker, Bremen, Germany) equipped with an electrospray ion source (ESI) operated in combined positive and negative ionization mode. Instrument parameters were optimized by infusion experiments with pure standards. The ion spray voltage was maintained at 5000 V and -4000 V in positive and negative ionization mode, respectively. Cone temperature was set to 300 °C and cone gas to 20 psi. Heated probe temperature was set to 200 °C and probe gas flow to 50 psi. Nebulizing gas was set to 60 psi and collision gas to 1.6 mTorr. Nitrogen was used as probe and nebulizing gas and argon as collision gas. Active exhaust was constantly on. Multiple reaction monitoring (MRM) was used to monitor analyte molecular ion → fragment ion transitions: MRM transitions were chosen based on direct infusion experiments. Detailed values for mass transitions can be found in Supplemental Table S2A. Both Q1 and Q3 quadrupoles were maintained at unit resolution. Alternatively, a modified MRM setting was used to select and filter the parent ion in both Q1 and Q3 while not applying any collision energy in Q2. This variation of Selected Ion Monitoring (SIM) was called SIM2 and increased the signal strength by approximately 3.5 to 15-fold and improved signal-to-noise ratio up to 10-fold compared to normal SIM where only Q1 is used for ion selection. Also for SIM2 both Q1 and Q3 were kept at unit resolution. Bruker MS Workstation software (Version 8.2, Bruker, Bremen, Germany) was used for data acquisition and processing. Linearity in ionization efficiencies were verified by analysing dilution
series of standard mixtures. Reference compounds for dhurrin, dhurrin amide, dhurrin acid, \( p \)-glucosyloxyphenylacetic acid (\( p \)GPAAc), \( p \)-hydroxyphenylacetic acid, \( p \)-hydroxyacetonitrile, and GS-pOHPACN were used for absolute quantification (nmol mg\(^{-1}\)) by using 12 points external calibration curves in a range of concentrations from 0.001 to 50 \( \mu \)M.

**Quantification of amino acids by LC-3Q-MS**

For the absolute quantification of free amino acids, the diluted sample was 1:10 mixed with \( ^{13} \mathrm{C}, \ ^{15} \mathrm{~N} \) labelled amino acids (Algal amino acids \( ^{13} \mathrm{C}, \ ^{15} \mathrm{~N} \), Isotec, Miamisburg, US) at a concentration of 10 \( \mu \)g mL\(^{-1}\). Diluted samples were filtered (Durapore\textsuperscript{®} 0.22 \( \mu \)m PVDF filters (Merck Millipore, Tullagreen, Ireland) and used directly for LC-MS analysis. The analysis was performed as described in (Mirza et al. 2016) with changes as detailed below. Briefly, chromatography was performed on an Advance UHPLC system (Bruker, Bremen, Germany). Separation was achieved on a Zorbax Eclipse XDB-C18 column (100 x 3.0 mm, 1.8 \( \mu \)m, Agilent Technologies, Germany). Formic acid (0.05% v/v) in water and acetonitrile (supplied with 0.05% v/v formic acid) were employed as mobile phases A and B, respectively. The elution profile was: 0-1.2 min 3% B; 1.2-4.3 min 3-65% B; 4.3-4.4 min 65-100% B; 4.4-4.9 min 100% B; 4.9-5.0 min 100-3% B; and 5.0-6.0 min 3% B. Mobile phase flow rate was 500 \( \mu \)L*min\(^{-1}\) and column temperature was maintained at 40\(^\circ\)C. The liquid chromatography was coupled to an EVOQ Elite TripleQuad mass spectrometer (Bruker, Bremen, Germany) equipped with an electrospray ionization source (ESI). Instrument parameters were optimized by infusion experiments with pure standards. The ion spray voltage was maintained at 3000 V in positive ion mode. Cone temperature was set to 300 \( ^\circ\)C and cone gas flow to 20 psi. Heated probe temperature was set to 400 \( ^\circ\)C and probe gas flow set to 50 psi. Nebulizing gas was set to 60 psi and collision gas to 1.6 mTorr. Nitrogen was used as both cone gas and nebulizing gas and argon as collision gas. Multiple reaction monitoring (MRM) was used to monitor analyte molecular ion \( \rightarrow \) fragment ion transitions: MRMs for amino acids were chosen as described in (Jander et al. 2004) with additions from (Docimo et al. 2012) for Arg and Lys. Both Q1 and Q3 quadrupoles were maintained at unit resolution. Bruker MS Workstation software (Version 8.2.1, Bruker, Bremen, Germany) was used for data acquisition and processing. Individual amino acids in the sample were quantified by the respective \( ^{13} \mathrm{C}, \ ^{15} \mathrm{~N} \)-labeled amino acid internal standard, except for tryptophan, and asparagine: tryptophan was quantified using \( ^{13} \mathrm{C}, \ ^{15} \mathrm{~N} \)-Phe applying a response factor of 0.42, asparagine was quantified using \( ^{13} \mathrm{C}, \ ^{15} \mathrm{~N} \)-Asp applying a response factor of 1.0 (Docimo et al. 2012). Further details for transitions and collision energies can be found in Supplemental Table S2B.

**Detection of dhurrin glucosides by LC-qTOF-MS**

Full scan analysis of sorghum extracts was performed using a Thermo Scientific Dionex Ultimate 3000 RS (ThermoFisher Scientific) coupled with a Bruker compact QqTOF Mass spectrometer (Bruker Daltonics) equipped with an electrospray ionization (ESI) source. For data acquisition and processing, Compass DataAnalysis software (version 4.3, Bruker Daltonics) was used. 8 \( \mu \)L of samples were injected on a LC Kinetex® XB-C18 column (150 x 2.1mm, 1.7\( \mu \)m, Phenomenex, Torrance, California, United States) and compound separation was achieved using a gradient of A = MilliQ grade water with 0.05 % v/v formic acid, and B = acetonitrile with 0.05 % v/v formic acid. The elution profile was 0-5 min, 2 % B; 4-9 min 30 % B; 9-14 min 100 % B; 14-21 min 100 %; 21-28 min 2 % B. The mobile phase flow rate was 300 \( \mu \)L*min\(^{-1}\) and column temperature was maintained at 40 \( ^\circ\)C. The full-scan mass spectra were obtained within a range of m/z 50-1300 with the instrument operated in the positive ion mode. The spray voltage was set at 4000 V with the maximum dry temperature set at 250 \( ^\circ\)C at the pressure of 1.2 Bar. The up-front collision-induced dissociation (CID) was set to off and the optimized collision energy was set at 7eV in the collision cell. The quadrupole...
mass filter was set with low mass and with the ion energy of 4 eV. Extracted ion chromatograms for specific [M+H]+ adduct ions were used to detect dhurrin glucosides. Their fragmentation patterns, accurate masses and retention times were used to identify peaks according to Pičmanová et al. (2015).

**MALDI-MSI sample preparation and fluorescence microscopy**

Samples were prepared according to Kawamoto (2003) method with slight modifications. Frozen samples were embedded into a 2 % w/v aqueous medium of carboxymethylcellulose (CMC) into a cryo-mold. The mold containing the embedded sample was submerged into a coolant solution of hexane/dry ice for approximately 5 min. When completely frozen, CMC blocks were removed from the mould and stored at -80 °C until cryo-sectioning. Frozen samples were transferred to a Leica CM3050S cryostat (Leica Microsystems, Wetzlar, Germany) at -30 °C to thermally equilibrate for 1 h. The embedded samples were fixed on the sample holder of the cryo-microtome using OTC gel (optimum cutting temperature, Tissue-Tek®), sectioned at 10 µm thickness, collected with cryofilm II C9 (Section Lab, Japan) which was attached to pre-chilled glass slides with double-sided adhesive carbon tape (Agar scientific, UK), and finally freeze-dried under vacuum at 1.0 mbar overnight. Three consecutive sections were selected for each sample; one was used for fluorescence microscopy analysis inspection and the others were coated with respectively DHB and DAN by sublimation, as described by Hankin et al. (2007). All the fluorescence images were captured on a Leica DMR HC (Leica Microsystems) wide-field fluorescence microscope, using a filter cube with a 340-380 nm excitation filter, 400 nm beam splitter and 420 nm long-pass emission filter. Autofluorescence exposure times varied based on the stage of the grain and the tissue composition (i.e. dry grain or germinated grain with protruded axes) but generally were set around between 600 and 800 ms. FIJI ImageJ (Schindelin et al., 2012) was used for any additional image preprocessing, such as cropping, adding the scale bar or other relevant operations.

**MALDI-MSI analyses**

MALDI-MSI analysis was performed on a Thermo QExactive Orbitrap mass spectrometer (Thermo Scientific, Bremen, Germany) equipped with the AP-SMALDI10 ion source (TransMIT, Giessen, Germany). The AP-SMALDI10 ion source was equipped with a MNL100 nitrogen laser (LTB Lasertechnik Berlin, Germany) with 337 µm wavelength and 60 Hz frequency, operated using 30 laser pulses per pixel. Spectra were acquired in positive and negative ion mode (scan range m/z 70-700) at mass resolving power 140,000 at m/z 200. A matrix peak was used as lock-mass for internal mass calibration, ensuring a mass accuracy of 2 ppm or better. The acquired raw files were converted to imzML files using an imzML converter (Schramm and al., 2012), and images were generated using MSiReader v.1.01 (MATLAB R2014b) (Robichaud and all, 2013) using a bin width of 0.01 Th and the following default parameters: MaxColorScale: 100,000, NormalizeOption: TIC, NormScale: 1 NormCutoff: 1, Minimum Abundance Threshold: 0.001. All MS images were normalized to the total ion count (TIC) in each pixel, and the maximum values for generating images are listed in Supplemental Fig. S4. The same colour scale was used regardless of genotype or germination stage. Metabolites were identified by accurate mass and the complete list of m/z values and the mass tolerance applied for each is available in supplementary information (Supplemental Table S3).
Supplemental data

The following supplemental materials are available.

**Supplemental Table S1.** The concentrations of dhurrin and dhurrin recycling products in four different lines.

**Supplemental Table S2.** Transitions and response factors used for Multiple Reaction Monitoring (MRM) for analysis by LC-3Q-MS.

**Supplemental Table S3.** Compounds visualized by MALDI-MSI.

**Supplemental Figure S1.** Concentration of GS-pOHPACN during germination and early seedling development in sorghum wild types.

**Supplemental Figure S2.** Dhurrin glucosides content during germination and early seedling development in sorghum wild types.

**Supplemental Figure S3.** Concentrations of individual amino during germination and early seedling development in four sorghum lines. **Supplemental Figure S4:** Maximum values for generating images.

**Supplemental Figure S5:** Distribution and compartmentalization of general metabolites in tcd1.

**Supplemental Figure S6:** Comparison of average raw mass spectra of two selected samples of BTx623 and tcd1 at stage S1.

**Supplemental Figure S7:** All replicates of images in Figure 6.

**Supplemental Figure S8:** Distribution of all detected amino acids during germination in BTx623 by MALDI-MSI

**Supplemental Figure S9:** Distribution of a subset of amino acids across biological replicates.

**ACKNOWLEDGMENTS**

We thank Professor Alexander Schulz, University of Copenhagen, for expert advice about fluorescence microscopy, Dr. Camilla Knudsen for helping out with the graphics of Fig. 2., and Dr. David Pattison for assistance with LC-qTOF-MS. L.M., N.B., M.S. M. thank the VELUX FOUNDATIONS [VKR023054 and 19151] and ERC Advanced Grant [323034] for support for this research. The Danish National Research Foundation [DNRF99] is acknowledged for the support provided to C.C. The development and characterisation of the tcd1 mutants was funded by Australian Research Council Grants to R.M.G (LP0774941, LP100100434, DP130101049) in partnership with Pacific Seeds Pty Ltd. Support from the Carlsberg Foundation and The Danish Council for Independent Research | Medical Sciences (grant no. DFF – 4002-00391) for MALDI-MSI instrumentation is also gratefully acknowledged.

**Figure Legends**

**Figure 1.** Structures of dhurrin and its identified recycling products found in sorghum. (1)

Dhurrin, (2) Dhurrin amide, (3) Dhurrin acid, (4) Dhurrin glucosides (representative structure, exact
linkage positions unknown), (5) p-hydroxyphenyl(S-glutathione)acetonitrile (GS-pOHPACN), (6) p-glucosyloxy phenylacetic acid (pGPAAc), (7) Caffeoyl-dhurrin, (8) Caffeyol-dhurrin acid.

Figure 2. Sorghum germination process: embryo and seedling development and sampling time points. A. Timeline of imbibition, germination and collection of samples. Sorghum grain were imbibed in water for 12 h and germinated for 72 h. Samples were collected every twelve hours at the desired time points, as indicated with arrows. All eight time points were subjected to metabolite profiling. From the eight time points, four developmental stages (S) were selected for analysis by Mass Spectrometry Imaging (MSI) and named: S0: dry seed, S1: after 12 h of imbibition, S2: emerged radicle and coleoptile, S3: developed shoot and primary root. B. Graphical representation of the stages collected for MSI analyses. For the BTx623 line these stages matched the indicated time points (for tcd1 see Figure 3). C. Schematic of longitudinal sections of the four developmental stages selected for MSI analysis, showing detailed morphological features and main compartments.

Figure 3. Levels of dhurrin and its recycling products during germination and early seedling development in four sorghum lines. Absolute quantification of dhurrin (1), dhurrin acid (3) and p-glucosyloxy phenylacetic acid (pGPAAc; 6) in wild types A. BTx623, B. TCD1, C. tcd1 parent line and the mutant D. tcd1 (note the different scale compared to A-C). For tcd1 pGPAAc was the only detected dhurrin related compound, and this was only detected at the last two time points (60-72 h AI). p-Hydroxyphenyl(S-glutathione)acetonitrile (GS-pOHPACN; 5) was detected in the wild types, however in minute concentrations which are therefore shown separately in Fig. S1. Data for semi-quantification of dhurrin glucosides (4) are shown in Fig. S2. Arrows in A and D indicate the time points where the two lines analysed by MSI had reached the different developmental stages S0-S2. Values are mean of n = 3 biological replicate for each time point. Exact values and the calculated standard errors are listed in Table S1.

Figure 4. Total free amino acids content during germination and early seedling development in four sorghum lines. The bars show the total sum of all free proteinaceous amino acids, except glycine which could not be quantified with the applied method but is usually present in low amounts. Data for individual amino acids are shown in Fig. S3. Values are mean of n = 3 replicates for each time point. The error bars represent the standard error within the biological replicates.

Figure 5. Distribution and compartmentalization of general metabolites during germination and early seedling development of the BTx623 wild type line. Three consecutive cryo-sections were imaged: The optical images on the far left (BTx623) were acquired with fluorescence microscopy and the remaining images using MALDI-MSI in both negative and positive ion mode, using respectively DAN and DHB as matrix at 30 µm spatial resolution. Developmental stages as in Fig. 2. The dashed lines define of the recorded MS images. A. Comparative distribution of hexose and dihexose using the two ionization modes. In positive mode compounds were detected as potassium adducts ([M+K]+), and in negative mode as deprotonated ions ([M-H]-). B. Specific tissue compartmentalization of choline [M+H]+ and phosphocholine (PC 0:16) [M+K]+. Scale bar = 1mm. Signals for all MSI images are normalized to TIC on each pixel and maximum values for generating images are listed in Fig. S4.
Figure 6. Distribution and compartmentalization of dhurrin and dhurrin recycling products during germination and early seedling development of the BTx623 wild type line. Procedures, settings and overall layout as in Fig. 5. All MS images were acquired in positive mode. Dhurrin was most consistently detected as a potassium adduct [M+K]+ and therefore the accurate mass of dhurrin potassium adducts (m/z 350.0636) was used to generate the images. Dhurrin glucosides were the only recycling products detected as a potassium adduct [M+K]+ whereas dhurrin acid, GS-pOHPACN and pGPAAc were detected as their sodium adducts [M+Na]+. MS images of tcd1 samples were blank for dhurrin (see Fig. S6) and its derivatives. All MS images were normalized to the total ion count in each pixel and maximum values for generating images are listed in Fig. S4. Scale bar = 1 mm.

Figure 7. Distribution of a selected subset of amino acids during germination and early seedling development in the BTx623 WT line and the mutant line tcd1. Procedures, settings and overall layout as in Fig. 5. A. BTx623. B. tcd1. Amino acids were all detected as deprotonated species, [M-H]-, in negative ionization mode. Scale bar = 1mm. A full data set of distributions of all amino acids in one replicate of BTx623 and in all analyzed replicates for the selected subset of amino acids in BTx623 and the mutant line tcd1 are provided in the supplementary data (respectively Fig. S8 and Fig. S9). All MS images were normalized to the total ion count in each pixel and maximum values for generating images are listed in Fig. S4.

Ahmad BK, Karim MA (1951) Biosynthesis of choline in the seedling of the chick pea (Cicer arietinum). Arch Neurol Psychiat.


PubMed: Author and Title
Google Scholar: Author Only Title Only Author and Title


PubMed: Author and Title
Google Scholar: Author Only Title Only Author and Title


PubMed: Author and Title
Google Scholar: Author Only Title Only Author and Title


PubMed: Author and Title
Google Scholar: Author Only Title Only Author and Title


PubMed: Author and Title
Google Scholar: Author Only Title Only Author and Title


PubMed: Author and Title
Google Scholar: Author Only Title Only Author and Title


PubMed: Author and Title
Google Scholar: Author Only Title Only Author and Title


PubMed: Author and Title
Google Scholar: Author Only Title Only Author and Title


PubMed: Author and Title
Google Scholar: Author Only Title Only Author and Title


PubMed: Author and Title
Google Scholar: Author Only Title Only Author and Title


PubMed: Author and Title
Google Scholar: Author Only Title Only Author and Title


PubMed: Author and Title
Google Scholar: Author Only Title Only Author and Title


PubMed: Author and Title
Google Scholar: Author Only Title Only Author and Title


PubMed: Author and Title
Google Scholar: Author Only Title Only Author and Title


PubMed: Author and Title
Google Scholar: Author Only Title Only Author and Title


PubMed: Author and Title
Google Scholar: Author Only Title Only Author and Title


Kope HH, Tsantrizos YS, Fortin JA, Ogilvie KK (1991) 3-Hydroxybenzoylformic acid and (R)-(−)-p-hydroxymandelic acid, two antifungal compounds isolated from the liquid culture of the ectomycorrhizal fungus Pisolithus arhizus. Can J Microbiol 37: 258–64.


Copyright (c) 2020 American Society of Plant Biologists. All rights reserved.


Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title