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# Counting the costs: nitrogen partitioning in *Sorghum* mutants

*Cecilia K. Blomstedt*[A], *Viviana C. Rosati*[A], *Birger Lindberg Møller*[B] *and Ros Gleadow*[A,C]

[A]School of Biological Sciences, Monash University, Clayton, Vic. 3800, Australia.
[B]Plant Biochemistry Laboratory and VILLUM Research Centre for Plant Plasticity, Department of Plant and Environmental Sciences, University of Copenhagen, 40 Thorvaldsensvej, DK-1871 Frederiksberg C, Copenhagen, Denmark.
[C]Corresponding author. Email: ros.gleadow@monash.edu

**Abstract.** Long-standing growth/defence theories state that the production of defence compounds come at a direct cost to primary metabolism when resources are limited. However, such trade-offs are inherently difficult to quantify. We compared the growth and nitrogen partitioning in wild type *Sorghum bicolor* (L.) Moench, which contains the cyanogenic glucoside dhurrin, with unique mutants that vary in dhurrin production. The *totally cyanide deficient 1* (*tcd1*) mutants do not synthesise dhurrin at all whereas mutants from the *adult cyanide deficient class 1* (*acdc1*) have decreasing concentrations as plants age. Sorghum lines were grown at three different concentrations of nitrogen. Growth, chemical analysis, physiological measurements and expression of key genes in biosynthesis and turnover were determined for leaves, stems and roots at four developmental stages. Nitrogen supply, ontogeny, tissue type and genotype were all important determinants of tissue nitrate and dhurrin concentration and turnover. The higher growth of *acdc1* plants strongly supports a growth/defence trade-off. By contrast, *tcd1* plants had slower growth early in development, suggesting that dhurrin synthesis and turnover may be beneficial for early seedling growth rather than being a cost. The relatively small trade-off between nitrate and dhurrin suggests these may be independently regulated.

**Additional keywords:** cyanogenesis, CYP79A1, defence, defense, dhurrin, nitrate, NIT4A/B2, resource allocation.

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## Introduction

To survive attack from pests and diseases plants have evolved effective and efficient defence systems including the production of a wide variety of specialised metabolites. Plant defence theories, such as the growth-differentiation balance hypothesis and optimal defence theory, are predicated on the assumption that there is a cost associated with diverting limited resources to processes that do not directly contribute to growth or reproduction (Herms and Mattson 1992; Cipollini *et al*. 2014). Costs are typically assessed by comparing the growth of plants that exhibit some degree of natural variation for the trait when supplied with different levels of resources (Züst and Agrawal 2017). However, the actual cost to the plants may be quite low in terms of energy and resources. Moreover, estimating the effect of resource supply on chemical composition may be confounded by the effect arising from reduced plant size (Miller *et al*. 2014).

One major group of chemical defence compounds are the cyanogenic glucosides (CNglcs) – nitrogen-containing compounds that may be hydrolysed to release toxic hydrogen cyanide (HCN) when mixed with degradative β-glucosidases in a process called cyanogenesis. Plants avoid autotoxicity by storing the CNglc and the hydrolysing enzyme in different locations. Cyanogenesis is common, found in ~10% of all plants from many different families, including over 30% of crops such as sorghum, apples, almonds and cassava (Jones 1998; Gleadow and Møller 2014).

Diverting resources to the synthesis of CNglcs should mean that cyanogenic plants grow more slowly than non-cyanogenic ones in the absence of herbivores, but perform comparatively better when herbivores are present. In practice, such experiments have yielded variable results, with some showing advantages and others disadvantages, in terms of biomass production, depending on the age and environmental conditions (Cipollini *et al*. 2014; Gleadow and Møller 2014). The majority of studies designed to test whether there is a growth cost to synthesising cyanogenic glucosides have used nitrogen-fixing legumes (e.g. Kakes 1989; Briggs and Schultz 1990). Given that nitrogen is essential for CNglc synthesis, source effects would be limited in such studies (Ballhorn *et al*. 2011). Another confounding variable is that trade-offs between costs and benefits are likely to change over time and with developmental stage (Boege and Marquis 2005; Miller *et al*. 2014). For example, Simon *et al*. (2010) found that highly cyanogenic *Eucalyptus cladocalyx* F.Muell. seedlings grew marginally more slowly than seedlings with lower concentrations but when ontogenetic effects were taken into account, such trade-offs were difficult to detect.

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The common view that CNglcs are primarily defence compounds has been brought into question, with several authors suggesting that CNglcs may play key roles in nitrogen turnover, storage, and transport, thus offsetting costs (Burke *et al.* 2013; Neilson *et al.* 2013; Ullmann-Zeunert *et al.* 2013; Gleadow and Møller 2014; Miller *et al.* 2014; Pičmanová *et al.* 2015). Such turnover may allow plants to manage their nitrogen, storing it when availability and photosynthetic rates are high, and releasing it again at crucial points in the life cycle to promote growth. HCN can also be detoxified, generating reduced nitrogen in the form of ammonia, aspartate and asparagine (Fig. 1), which can be used in primary metabolism. Synthesis and turnover of CNglcs may also mitigate stresses by reducing reactive oxygen species (ROS) associated with drought (Selmar and Kleinwächter 2013; Burke *et al.* 2015) and may even be a source of carbon (e.g. Kongsawadworakul *et al.* 2009). In *Prunus* species, cyanogenic glucoside formation and turnover are involved in controlling floral bud break (Del Cueto *et al.* 2017; Ionescu *et al.* 2017*a*, 2017*b*).

Here, we compare the growth and chemical composition of mutants of *Sorghum bicolor* (L.) Moench that vary in their capacity to synthesise CNglcs, and that have access to different levels of nitrogenous fertiliser over time. Sorghum produces the CNglc dhurrin ((*S*)-4-hydroxymandelonitrile-β-D-glucopyranoside), which is derived from the amino acid tyrosine (Gleadow and Møller 2014). *S. bicolor* is a drought tolerant $C_4$ grass grown for grain, forage and, more recently, for biogas and ethanol production. Young plants contain extremely high concentrations of dhurrin (e.g. >10% dry mass), which decreases over the first few weeks of growth (Gleadow and Møller 2014). However, concentrations can increase again if plants are drought stressed or over fertilised (Burke *et al.* 2015; Neilson *et al.* 2015). In addition, sorghum stores excess nitrogen as nitrates. There is some evidence of a trade-off in the partitioning of nitrogen between nitrate and dhurrin, with this more likely during and after stress, and between different tissues (Gleadow *et al.* 2016). As both dhurrin and nitrates can be toxic to cattle (Finnie *et al.* 2011), a decrease in dhurrin that results in an increased nitrate concentration may ultimately be detrimental to grazing animals.

Rather than using natural variation, two sorghum mutants with altered cyanogenic status, identified from our EMS TILLING population (Blomstedt *et al.* 2012), were utilised in comparison to the non-mutated parent *S. bicolor* (Sb). One mutant is *totally cyanide deficient* (*tcd1*) and the other is thought to be a developmentally regulated mutant, in that the shoot tissue is only cyanogenic when plants are young and thus called the *adult cyanide deficient class1* mutant (*acdc1*). These mutants were identified using a qualitative biochemical screen of a mutant population derived from EMS treatment of sorghum seeds, followed by the reverse genetic technique of ‘targeted induced local lesions in genomes’ (TILLING) (Blomstedt *et al.* 2012). Detailed biochemical analyses of *tcd1* showed that CYP79A1 (the first enzyme in the biosynthetic pathway of dhurrin) is present in wild-type amounts but is catalytically inactive due to a nucleotide change resulting in a proline to leucine amino acid change substitution in the encoded protein (Blomstedt *et al.* 2012). Sequence analysis of the coding regions of the three genes in the dhurrin biosynthesis pathway (*CYP79A1*, *CYP71E1* and *UGT85B1*) of the second mutant, *acdc1*, identified no nucleotide changes. However, a C-to-T change consistent with EMS treatment has been identified ~1.1 kb upstream of the start codon of *CYP79A1* (CK Blomstedt, unpubl. data). Both these mutant lines have been backcrossed three times to BTx623 sorghum to minimise additional background mutations and

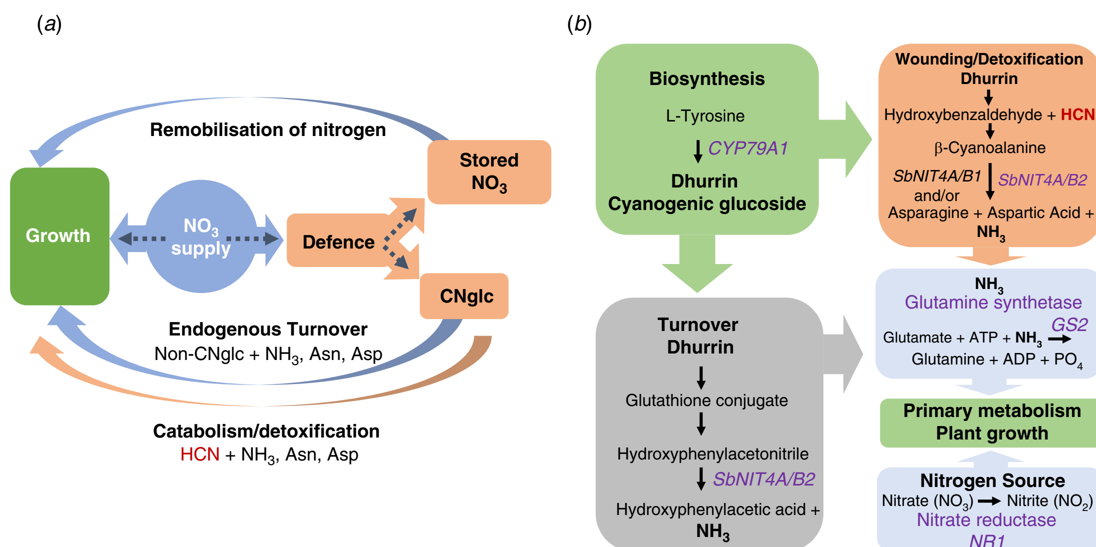



**Fig. 1.** (*a*) Partitioning of nitrogen between growth and defence in cyanogenic plants; (*b*) genes involved in the synthesis and turnover of dhurrin and nitrogen metabolism in *Sorghum*. Nitrogen, shown here as nitrate, can be used for either growth or defence. In sorghum, nitrate can either be reduced to dhurrin or stored as nitrate, sometimes in concentrations high enough to be toxic to grazing animals. Stored nitrate is easily mobilised, with the balance dependent on environmental and developmental factors (Gleadow *et al.* 2016). Cyanogenic glucosides may be detoxified and the reduced nitrogen used for growth in two ways: via the breakdown of toxic hydrogen cyanide, or the newly proposed alternative endogenous turnover pathway that does not involve the release of free hydrogen cyanide. Abbreviations: Asn, asparagine; Asp, aspartic acid; $NO_3$, nitrate; CNglc, cyanogenic glucosides.

appear phenotypically normal compared with wild type BTx623, except for the altered cyanogenic status. The growth of a third acyanogenic mutant line, which has a mutation in the gene governing the final glycosylation step, was not included in this study because its growth was impaired (Blomstedt *et al*. 2016).

The cyanide deficient mutants provide a unique system for measuring resource allocation by allowing nitrogen to be titrated against growth by manipulating both source and sink (Fig. 1*a*). This study is comprehensive and unique in that it investigates the effects of both source (nutrient availability and plant ontogeny) and sink (distinct genotypes varying in CNglc production) on growth and nitrogen allocation. In addition, quantitative PCR is used to determine the transcript levels of key genes involved in dhurrin synthesis (cytochrome P450, *CYP79A1*) and turnover (nitrilase, *NIT4B2*), and nitrogen assimilation (nitrate reductase 1, *NR1*; glutamine synthetase 2, *GS2*). These genes were chosen based on the key roles they play in the synthesis and turnover of dhurrin and nitrogen metabolism (Fig. 1*b*). The biosynthetic gene *CYP79A1* encodes the enzyme catalysing the first committed step in dhurrin biosynthesis. At some stages of sorghum ontogeny, dhurrin synthesis is under transcriptional control (Busk and Møller 2002; Nielsen *et al*. 2016). Jenrich *et al*. (2007) showed that the nitrilases, SbNIT4A, SbNIT4B1 and SbNIT4B2, form heteroduplexes that are involved in the detoxification of HCN (SbNIT4A/B1 and/or SbNIT4A/4B2), whilst only SbNIT4A/4B2 forms an active complex in the alternative nitrogen turnover pathway (Fig. 1*b*). Therefore, analysis of the expression level of *NIT4B2* may indicate if turnover is also transcriptionally regulated. Nitrate reductase (*NR1*) and glutamine synthetase (*GS2*) are important components of the nitrogen uptake and assimilation process. Nitrate is taken up by the roots and most is transported to the leaves where nitrate reductase reduces nitrate to nitrite and then to $NH_4^+$ via nitrite reductase, whilst GS2 recycles $NH_4^+/NH_3$ to form the key amino acid, glutamine (McAllister *et al*. 2012; Xu *et al*. 2012). *NR1* and *GS2* have previously been identified as differentially expressed among sorghum genotypes grown under nitrogen stress conditions (Gelli *et al*. 2014).

We tested two hypotheses: (i) if defence is costly, then plants that allocate nitrogen to dhurrin will have reduced growth rates when nitrogen supply is limited (Fig. 1); or conversely, if dhurrin is important for growth and development, then the acyanogenic mutants (*tcd1* and *acdc1*) will have a growth disadvantage; and (ii) if there is a trade-off in the proportion of nitrogen allocated to dhurrin and nitrate, then dhurrin deficient mutants will have higher concentrations of nitrate. Miller *et al*. (2014) showed that in relation to sorghum much of the difference in allocation of nitrogen to dhurrin could be accounted for by ontogenic differences when plants were grown under various nutrient regimes. Therefore, to avoid the effect of ontogeny, plants were harvested at specific stages of plant growth rather than chronological age (Vanderlip and Reeves 1972).

## Materials and methods

### *Plant material and growth conditions*

Three lines of sorghum were used to analyse the effects of nitrogen on growth, metabolites, and gene expression: a control line *Sorghum bicolor* (L.) Moench (Sb); and two mutant lines: *adult cyanide deficient class 1* (*acdc1*); and *totally cyanide deficient 1* (*tcd1*) (Blomstedt *et al*. 2012). The plants were grown in the greenhouse complex at Monash University, Clayton, Vic., Australia, from June to September 2013. The greenhouse received natural light, and the photoperiod was extended to 14 h using sodium lamps (MK-1 Just-a-shade, Ablite Australia). Air temperature, humidity and light intensity were recorded every 5 s (Humidex, Nelan Industries). The average photosynthetic photon flux density (0600–1800 hours) over the duration of the experiment was $444 \pm 92$ µmol quanta $m^{-2}\,s^{-1}$. Temperatures ranged from 15°C to 31°C with an average of $26 \pm 4$°C/$20 \pm 3$°C day/night (±s.e.). Average RH was $70 \pm 8$%/$81 \pm 8$% day/night (±s.e.).

Seeds of each line were germinated in seed trays on Debco seed raising substrate and perlite (2 : 1, v/v; Debco Pty Ltd). At the two-leaf stage, 70 individual plants of lines Sb and *acdc1*, and 40 of line *tcd1* were transferred to individual 20 cm pots containing ~3.8 L seed raising: perlite mix (as above). Fewer *tcd1* plants were used due to a lower rate of germination in comparison to the other two lines.

At the three-leaf stage the nitrogen treatments were applied as modified half-strength Long Ashton's solution (Miller *et al*. 2014) containing either a low (LN, 2 mM), medium (MN, 6 mM) or high (HN, 12 mM) level of nitrogen, with equivalent proportions of $NO_3^-$ and $NH_4^+$ (3 : 1, mol $mol^{-1}$). Plants were watered with 50 mL nutrient solution (containing the appropriate level of nitrogen) three times a week until the six-leaf stage, when it was increased to 100 mL. Throughout the experiment all plants were watered to saturation once a week with plain water to prevent accumulation of salts in the pots. The concentrations of nitrogen were chosen to ensure that supply was either limited, adequate or in excess of growth requirements (O'Donnell 2012).

Sorghum development and growth stages were defined by the number of leaves present, following Vanderlip and Reeves (1972). Plants with leaf numbers up to five are in the early vegetative growth phase, plants with eight leaves are at the transition from vegetative to the initiation of flowering, whilst plants with 10 leaves are about to boot (Vanderlip and Reeves 1972). Therefore, plants were harvested at the three-, five-, eight- and 10-leaf stages (three-leaf, five-leaf, eight-leaf and 10-leaf), rather than at particular times. Seven plants of each of the lines Sb and *acdc1*, and four of *tcd1* were harvested at each stage and for each nitrogen treatment. Plants at the three-leaf stage were harvested before the commencement of nitrogen treatments. Leaf area was measured using the LI-3000 portable area meter and LI-3050A belt conveyor (LI-COR BioSciences). Leaf and root samples for RNA extraction (~0.1 g) were snap frozen in liquid nitrogen and stored at −80°C until required. All other tissue was dried in an oven at 50°C for 7 days, at which point DW was determined and the tissue ground, using a MixerMill (MM 300, Retsch) or a coffee grinder (yellow-line, IKA-Werke) for larger samples.

### *Growth indices*

Growth parameters – relative growth rate (RGR), net assimilation rate (NAR), leaf area ratio (LAR), specific leaf area (SLA) and specific leaf nitrogen (SLN) – were derived from the harvest data

using the following equations, after Neilson *et al.* (2015) and Gleadow and Rowan (1982):

$$\text{RGR day}^{-1} = \frac{\ln W_2 - \ln W_1}{t_2 - t_1}, \quad (1)$$

$$\text{NAR}\,(\text{g m}^{-2}\,\text{day}^{-1}) = \left(\frac{W_2 - W_1}{t_2 - t_1}\right)\left(\frac{\ln A_2 - \ln A_1}{t_2 - t_1}\right), \quad (2)$$

$$\text{LAR}\,(\text{m}^2\,\text{g}^{-1}) = \frac{A}{W}, \quad (3)$$

$$\text{SLA}\,(\text{m}^2\,\text{g}^{-1}) = \frac{A}{W_L}, \quad (4)$$

and

$$\text{SLN g g}^{-1} = (N)\left(\frac{W_L}{A}\right), \quad (5)$$

where $W$ is total biomass, $W_L$ is leaf biomass, $A$ is leaf area, $t$ is time and $N$ is leaf nitrogen concentration.

### Chemical assays

All analyses were performed using finely ground leaf, sheath and root tissue. Hydrogen cyanide potential (HCNp) was determined using 10 mg of tissue, following Gleadow *et al.* (2012). HCNp is the total amount of HCN evolved from hydrolysis of the entire content of endogenous cyanogenic glucosides. It is used as a proxy for dhurrin, such that each mg of HCN is equivalent to 11.5 mg of dhurrin in the plant tissue. Total nitrate concentration was measured via a colourimetric assay in 96 well microtiter plates using 15 mg of tissue per sample (O'Donnell *et al.* 2013). Total nitrogen of the leaf, sheath and root samples was analysed by the Environmental Analysis Laboratory (Lismore, NSW, Australia). Three replicates of each tissue (from the different nitrogen levels, for each harvest time) were analysed for total nitrogen, except for the three-leaf stage where tissue was pooled due to the small plant size.

The total amount of nitrogen, dhurrin and nitrate per plant were calculated by multiplying concentration by biomass of each tissue type. In order to assess how nitrogen was partitioned, the proportion of nitrogen found as dhurrin or nitrate was calculated as a proportion of total elemental nitrogen on both tissue and whole plant bases.

Chlorophyll *a* and *b* and carotenoid levels were measured following the method by Burns *et al.* (2002). Leaf discs (~1.5 cm diameter) were taken from the centre of the youngest fully unfurled leaf, frozen in liquid nitrogen and stored at −20°C. Chlorophyll was extracted by grinding the leaf discs in ice-cold 80% acetone (v/v), centrifuging for 3 min at 10 600*g*, and collecting the supernatant for analysis. Absorbance was read at 450, 647 and 664 nm, and concentrations expressed based on fresh weight (mg $g^{-1}$ FW) (Burns *et al.* 2002).

### RNA extraction and cDNA synthesis

Tissue was ground for three cycles of 40 s using a FastPrep-24 Instrument (MP Biomedicals) cooled with liquid nitrogen. RNA was extracted from the leaves and roots using a Sigma Spectrum Plant Total RNA Kit (Sigma-Aldrich). The concentration was determined using a NanoDrop spectrophotometer (ND-1000, Thermo Fisher Scientific) and the quality checked by running the RNA on an 8% formaldehyde gel. Complementary DNA was synthesised from 1 µg total RNA using SuperScript III First-Strand Synthesis System for RT–PCR (Invitrogen) and an oligo dT primer for three plant replicates of both the leaves and roots for each line, nitrogen level and harvest time. The cDNA was then used to determine the expression of selected genes using quantitative PCR (qPCR).

### Quantitative PCR

Transcript levels of *CYP79A1* (Sb01g001200), *NIT4B2* (Sb04 g026940), *nitrate reductase 1* (*NR1*, Sb07g022750) and *glutamine synthetase 2* (*GS2*, Sb06g031460) normalised to *ubiquitin* (*UBT*, Sb01g030340) (Paolacci *et al.* 2009) were determined by qPCR using a Light Cycler 480II (Roche) for three replicates of both the leaves and roots of each line (Sb, *acdc1* and *tcd1*), nitrogen level (LN, MN and HN) and leaf stage (three-, five-, eight- and 10-leaf) (Fig. 1*b*). The sorghum genome has been sequenced (Paterson *et al.* 2009) providing a resource to identify the sequences of the selected genes. Forward and reverse primers (Sigma-Aldrich), specific for a region in the 3′ end of each gene transcript sequence, were designed using PerlPrimer (http://perlprimer.sourceforge.net/; see Table S1, available as Supplementary Material to this paper). The Epmotion 5075 Robot (Eppendorf) was used to set up the 384 well plates. SensiMix SYBR Green No ROX kit (Bioline) was used for qPCR according to the manufacturer's instructions. Each sample was run in triplicate along with a set of standards specific for the gene being analysed. Each RNA preparation (i.e. the RNA used to create the cDNA) was also checked for DNA contamination by using crude RNA as the template and determining the occurrence of any amplification (data not shown).

### Statistical analysis

The statistics package SigmaPlot ver. 12.2 (Systat Software) was used for statistical analyses by one- or two-way ANOVA. For all tests, a *P*-value of <0.05 was considered significant. When an interaction was detected, *post-hoc* comparisons were undertaken using Tukey's test.

## Results

### Growth differences in cyanogenic and acyanogenic genotypes and interactions with N supply

Wild-type and mutant sorghum lines that produce differing amounts of the cyanogenic glucoside, dhurrin, were grown at three different levels of nitrogen fertiliser (LN, 2 mM; MN, 6 mM; HN, 12 mM) for up to 17 weeks. Leaf area, height and biomass of plants were measured at common leaf stages (see Fig. S1, available as Supplementary Material to this paper) to reduce any confounding effects of developmental stage on the concentration of dhurrin. Two key observations were made. First, nitrogen was the most important determinant of plant size at all growth stages, with the strongest effect observed in the leaf area of plants at the eight-leaf stage (Table S2). Second, there were significant genotype effects: *tcd1* plants were shorter than both *acdc1* and Sb plants throughout the experiment, but leaf area and

total biomass of *tcd1* plants were generally not significantly different or greater than the other two lines (Table S2).

Relative growth rate (RGR) was calculated at each harvest point (Table S2) and for the entire growing period (Fig. 2). Overall the primary driver of RGR was nitrogen supply, with significant reductions in RGR corresponding to the decreasing nitrogen supply over time (Fig. 2; Table S2). In terms of genotype, the totally cyanide deficient line (*tcd1*) had a significantly higher overall RGR compared with the wild-type Sb line at MN (6 mM; MN; $P < 0.001$, $F_{2,45} = 16.1$; Fig. 2).

Differences in morphological traits between the three genotypes and in response to different nitrogen treatments were observed over the course of the experiment (Figs 2, S1; Table S2). Early effects were predominantly genotype-driven. At the three-leaf stage there was a significant difference between genotypes for height ($P < 0.001$, $F_{2,15} = 19.1$), with the totally acyanogenic line, *tcd1*, being significantly shorter than *acdc1*, and both mutants significantly shorter than the wild-type Sb line (Table S2a). At the five-leaf stage, when plants had been treated with different levels of nitrogen for ~2 weeks, there was virtually no effect of treatment on plant growth (Table S2b). However, there were genotype differences: *tcd1* plants were again shorter (by ~20%) regardless of the level of nitrogen applied ($P < 0.001$, $F_{2,45} = 30.5$), but the leaf area of *tcd1* was higher than both Sb and *acdc1* ($P = 0.002$, $F_{2,45} = 6.9$).

At the eight-leaf stage the primary driver of differences in growth remained nitrogen supply with successive and significant reductions in height ($P < 0.001$, $F_{2,45} = 12.4$) and leaf area ($P < 0.001$, $F_{2,45} = 18.9$) in plants grown under LN (2 mM, LN) compared with MN (6 mM, MN) and HN (12 mM, HN) treatments (Table S2c). At this developmental stage, the main genotypic difference was that *acdc1* had a significantly greater leaf area ($P = 0.001$, $F_{2,45} = 7.7$) and total biomass ($P < 0.001$, $F_{2,45} = 8.5$) at HN and MN compared with both Sb and *tcd1* plants (Table S2c). The *acdc1* plants were also taller than both Sb and *tcd1*. When plants were grown under LN, however, there was no significant difference in leaf area or total biomass among the three genotypes (Table S2c). At the final harvest (10-leaf stage) the main differences were again due to the nitrogen treatments, with significant reductions in leaf area ($P < 0.001$, $F_{2,45} = 16.4$) and height ($P < 0.001$, $F_{2,45} = 21.5$) with decreased nitrogen application (Table S2d). The *acdc1* plants were again taller than both Sb and *tcd1* ($P < 0.001$, $F_{2,45} = 16.1$) with an equivalent leaf area (Table S2d). No significant differences between the three genotypes in response to differing nitrogen treatments with respect to total plant biomass were detected.

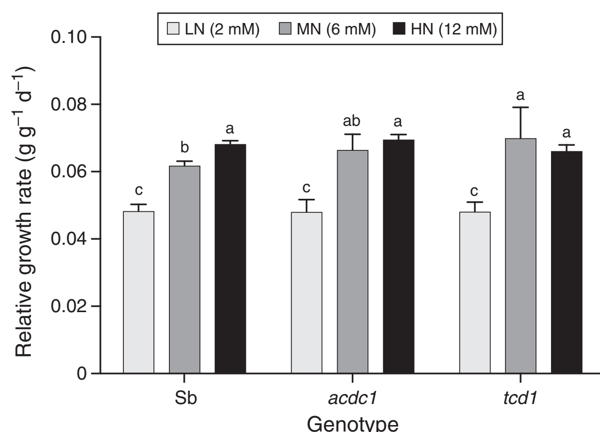



**Fig. 2.** Overall relative growth rate of wild type *Sorghum bicolor* (Sb) and mutants that are either totally acyanogenic (*tcd1*) or acyanogenic as adults (*acdc1*) and grown at three concentrations of nitrogen (2, 6 and 12 mM; low nitrogen (LN), medium nitrogen (MN) and high nitrogen (HN) respectively). Values are means ± s.e. ($n = 7$ for Sb and *acdc1*, $n = 4$ for *tcd1*). Columns with different letters are significantly different at $P < 0.05$, analysed using ANOVA and Tukey's test. Data here represent growth of plants between the three-leaf and 10-leaf stages of development. A more detailed breakdown of growth for each stage is given in Table S2, available as Supplementary Material to this paper.

### *Plant composition: tissue and age dependent allocation of nitrogen to nitrate and dhurrin*

Hydrogen cyanide potential (HCNp $mg\,g^{-1}$), nitrate ($NO_3^-$ $mg\,g^{-1}$) and total elemental nitrogen (%) were determined for all lines, for all treatments and at all time points (Tables 1–3). HCNp was primarily dependent on genotype. Nitrogen level also affected HCNp, but the effects were only significant at the later stages of development (Tables 1–3; Fig. S2). For example, Sb and *acdc1* plants grown at HN had equivalent HCNp at the eight-leaf stage (Table 1). Nitrogen was a significant driver of $NO_3^-$ concentration, and total $NO_3^-$ content per plant (i.e. per total biomass) (Table 2). There was little difference in the total elemental nitrogen (%) or total plant nitrogen (mg) between the lines. There were, however, significant treatment effects with progressively lower total nitrogen with decreasing nitrogen supply (Table 3; $P < 0.001$). In comparison to the roots, almost twice the amount (%) of total nitrogen was found in the leaves and sheaths of the plants at all developmental stages ($P < 0.001$). This is important as it correlates with the higher concentrations of hydrogen cyanide and $NO_3^-$ in the above ground plant tissues.

The *tcd1* mutants were completely acyanogenic at all stages and treatments (Table 1). The HCNp of the *acdc1* mutants was significantly lower (70–75%) than the Sb plants at the three-leaf stage. The age-dependent decrease in HCNp was also much faster in the *acdc1* plants than Sb, such that HCNp was a further 5–10% lower in plants harvested at the five-leaf stage. By the eight-leaf stage HCNp was approaching zero in the above ground tissues of *acdc1* at all nitrogen treatments, except HN (Table 1). In the root tissue, by contrast, there was no significant difference between HCNp in Sb and *acdc1* until the 10-leaf stage where HCNp in the *acdc1* roots decreased to approximately half that seen in the Sb plants with concentrations of $0.20 \pm 0.04\,mg\,g^{-1}$ dry weight compared with $0.44 \pm 0.04\,mg\,g^{-1}$ DW in plants from the MN treatment (Table 1). Calculations of the total HCNp per plant (rather than per mass) were consistent with these data, with significantly lower levels in *acdc1* (Table 1).

Nitrate concentrations were highly dependent on nitrogen supply. The $NO_3^-$ concentration of LN grown plants was less than half that of plants grown at higher levels of N (Table 2). Tissue type was also an important determinant of $NO_3^-$ concentration with concentration in the sheaths being consistently around 70–80 $mg\,g^{-1}$ DW, compared with 20 $mg\,g^{-1}$ DW in the roots and leaves when grown under high nitrogen

**Table 1. Hydrogen cyanide potential (HCNp mg $g^{-1}$ DW), whole plant cyanide (mg) of the leaf, sheath and root tissue of *Sorghum bicolor* (Sb) and two cyanide deficient mutants (*acdc1* and *tcd1*)**
Plants were grown at three concentrations of nitrogen: high (HN, 12 mM), medium (MN, 6 mM) and low (LN, 2 mM) and harvested at four leaf stages. Nitrogen treatments were imposed after the first harvest. Values are means ± s.e. ($n = 7$ for Sb and *acdc1*, $n = 4$ for *tcd1*). Total hydrogen cyanide per plant (HCN mg) was calculated by multiplying the concentration by the mass for each tissue. Significance of one-way (three-leaf stage) or two-way ANOVAs (other leaf stages) are presented for each genotype (line, L) and treatment (T): *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not significant. Means with the same lower case letter are not significantly different ($P > 0.05$; Tukey's pairwise comparison)

| | Sb | | | *acdc1* | | | *tcd1* | | | ANOVA | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | LN | MN | HN | LN | MN | HN | LN | MN | HN | L | T | L × T |
| | | | | | *Three-leaf stage* | | | | | | | |
| Leaf | | 3.12 ± 0.23a | | | 0.97 ± 0.33b | | | 0.00 ± 0.00c | | *** | | |
| Sheath | | 1.49 ± 0.17a | | | 0.38 ± 0.09b | | | 0.00 ± 0.00b | | *** | | |
| Root | | 1.34 ± 0.19a | | | 0.89 ± 0.20a | | | 0.00 ± 0.00b | | *** | | |
| HCN per plant (mg) | | 0.12 ± 0.05a | | | 0.05 ± 0.02b | | | 0.00 ± 0.00c | | *** | | |
| | | | | | *Five-leaf stage* | | | | | | | |
| Leaf | 1.12 ± 0.06b | 0.85 ± 0.10a | 0.84 ± 0.09a | 0.23 ± 0.03c | 0.39 ± 0.23c | 0.20 ± 0.05c | 0.00 ± 0.00d | 0.00 ± 0.00d | 0.00 ± 0.00d | *** | ns | * |
| Sheath | 0.32 ± 0.06 | 0.29 ± 0.05 | 0.37 ± 0.04 | 0.14 ± 0.04 | 0.15 ± 0.03 | 0.19 ± 0.03 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 | *** | ns | ns |
| Root | 0.36 ± 0.08 | 0.50 ± 0.09 | 0.43 ± 0.04 | 0.34 ± 0.01 | 0.31 ± 0.08 | 0.36 ± 0.03 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 | *** | ns | ns |
| HCN per plant (mg) | 0.12 ± 0.02 | 0.12 ± 0.03 | 0.14 ± 0.03 | 0.04 ± 0.01 | 0.07 ± 0.01 | 0.05 ± 0.01 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 | *** | ns | ns |
| | | | | | *Eight-leaf stage* | | | | | | | |
| Leaf | 0.25 ± 0.01a | 0.32 ± 0.04ab | 0.33 0.02ab | 0.01 ± 0.00c | 0.01 ± 0.00c | 0.32 ± 0.02b | 0.00 ± 0.00c | 0.00 ± 0.00c | 0.00 ± 0.00c | *** | ** | * |
| Sheath | 0.14 ± 0.02b | 0.13 ± 0.02b | 0.07 ± 0.02a | 0.03 ± 0.01c | 0.05 ± 0.01c | 0.05 ± 0.01ac | 0.00 ± 0.00c | 0.00 ± 0.00c | 0.00 ± 0.00c | *** | ns | * |
| Root | 0.28 ± 0.04 | 0.37 ± 0.06 | 0.30 ± 0.05 | 0.23 ± 0.06 | 0.23 ± 0.07 | 0.22 ± 0.07 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 | *** | ns | ns |
| HCN per plant (mg) | 0.18 ± 0.04b | 0.20 ± 0.08ab | 0.25 ± 0.05a | 0.06 ± 0.04c | 0.07 ± 0.04c | 0.29 ± 0.08a | 0.00 ± 0.00c | 0.00 ± 0.00c | 0.00 ± 0.00c | *** | *** | *** |
| | | | | | *10-leaf stage* | | | | | | | |
| Leaf | 0.43 ± 0.02a | 0.45 ± 0.03a | 0.32 ± 0.03b | 0.09 ± 0.08c | 0.05 ± 0.02c | 0.02 ± 0.01c | 0.00 ± 0.00c | 0.00 ± 0.00c | 0.00 ± 0.00c | *** | * | ** |
| Sheath | 0.07 ± 0.0a | 0.04 ± 0.00b | 0.07 ± 0.01a | 0.01 ± 0.00bc | 0.03 ± 0.02bc | 0.04 ± 0.02b | 0.00 ± 0.00c | 0.00 ± 0.00c | 0.00 ± 0.00c | *** | ns | * |
| Root | 0.26 ± 0.02 | 0.44 ± 0.04 | 0.44 ± 0.03 | 0.09 ± 0.02 | 0.20 ± 0.04 | 0.24 ± 0.02 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 | *** | *** | ns |
| HCN per plant (mg) | 1.06 ± 0.28b | 0.98 ± 0.31b | 0.70 ± 0.21a | 0.09 ± 0.08c | 0.29 ± 0.13c | 0.34 ± 0.25c | 0.00 ± 0.00cd | 0.00 ± 0.00cd | 0.00 ± 0.00d | *** | ns | ** |

**Table 2. Nitrate concentration ($NO_3^-$ mg $g^{-1}$ DW) and whole plant $NO_3^-$ (mg) of the leaf, sheath and root tissue of *Sorghum bicolor* (Sb) and two cyanide deficient mutants (*acdc1* and *tcd1*)**

Plants were grown at three concentrations of nitrogen: high (HN, 12 mM), medium (MN, 6 mM) and low (LN, 2 mM) and harvested at four leaf stages. Nitrogen treatments were imposed after the first harvest. Values are means ± s.e. ($n = 7$ for Sb and *acdc1*, $n = 4$ for *tcd1*). Total nitrate per plant ($NO_3^-$ mg) was calculated by multiplying the concentration by the mass for each tissue. Significance of one-way (three-leaf stage) or two-way ANOVAs (other leaf stages) are presented for each genotype (line, L) and treatment (T): *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not significant. Means with the same lower case letter are not significantly different ($P > 0.05$; Tukey's pairwise comparison)

| | Sb | | | *acdc1* | | | *tcd1* | | | ANOVA | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | LN | MN | HN | LN | MN | HN | LN | MN | HN | L | T | L × T |
| | | | | | *Three-leaf stage* | | | | | | | |
| Leaf | | 26 ± 1 | | | 33 ± 7 | | | 42 ± 7 | | ns | | |
| Sheath | | 74 ± 3 | | | 69 ± 7 | | | 71 ± 10 | | ns | | |
| Root | | 29 ± 6 | | | 31 ± 6 | | | 36 ± 5 | | ns | | |
| $NO_3^-$ per plant (mg) | | 3 ± 1 | | | 2 ± 1 | | | 3 ± 1 | | ns | | |
| | | | | | *Five-leaf stage* | | | | | | | |
| Leaf | 16 ± 1 | 25 ± 1 | 25 ± 1 | 18 ± 3 | 21 ± 2 | 24 ± 4 | 13 ± 5 | 21 ± 2 | 23 ± 1 | ns | *** | ns |
| Sheath | 62 ± 3 | 77 ± 2 | 76 ± 7 | 62 ± 10 | 80 ± 8 | 81 ± 7 | 45 ± 17 | 86 ± 4 | 84 ± 6 | ns | *** | ns |
| Root | 14 ± 2 | 19 ± 1 | 23 ± 1 | 16 ± 3 | 19 ± 2 | 25 ± 2 | 6 ± 4 | 19 ± 4 | 22 ± 1 | ns | *** | ns |
| $NO_3^-$ per plant (mg) | 4 ± 1 | 7 ± 0.4 | 9 ± 2 | 5 ± 3 | 7 ± 2 | 8 ± 3 | 4 ± 3 | 10 ± 4 | 9 ± 2 | ns | *** | ns |
| | | | | | *Eight-leaf stage* | | | | | | | |
| Leaf | 2 ± 0.2c | 19 ± 1a | 26 ± 1a | 3 ± 1c | 9 ± 1bc | 14 ± 1b | 19 ± 8a | 24 ± 5a | 19 ± 1ab | *** | *** | *** |
| Sheath | 4 ± 1d | 64 ± 3b | 95 ± 5a | 3 ± 1d | 82 ± 11ab | 82 ± 6a | 9 ± 3d | 28 ± 2.7cd | 38 ± 3c | *** | *** | *** |
| Root | 0.1 ± 0.1c | 9 ± 2b | 19 ± 1a | 7 ± 3de | 2 ± 1e | 9 ± 1d | 3 ± 2cd | 12 ± 3b | 21 ± 1a | ** | *** | *** |
| $NO_3^-$ per plant (mg) | 2 ± 0.6c | 21 ± 2bd | 45 ± 10a | 3 ± 3c | 29 ± 11d | 38 ± 7a | 10 ± 6c | 13 ± 6cb | 27 ± 9e | * | *** | *** |
| | | | | | *10-leaf stage* | | | | | | | |
| Leaf | 6 ± 3 | 8 ± 1 | 20 ± 1 | 3 ± 0.4 | 5 ± 1 | 13 ± 1 | 4 ± 0.5 | 6 ± 1 | 20 ± 4 | ** | *** | ns |
| Sheath | 6 ± 1b | 19 ± 5b | 81 ± 10a | 3 ± 0.5b | 38 ± 8bc | 55 ± 9c | 3 ± 1b | 19 ± 8b | 74 ± 4ac | ns | *** | * |
| Root | 2 ± 0.3b | 4 ± 1b | 19 ± 3a | 2 ± 0.5b | 2 ± 0.5b | 10 ± 1c | 2 ± 0.3b | 2 ± 0.5b | 17 ± 1ac | * | *** | * |
| $NO_3^-$ per plant (mg) | 19 ± 14b | 27 ± 11bd | 97 ± 24a | 7 ± 1b | 42 ± 8d | 69 ± 21c | 8 ± 4b | 20 ± 7b | 78 ± 10ac | ns | *** | * |

($P < 0.001$; Table 2). There were no significant differences between genotypes for $NO_3^-$ at the three- and five-leaf stages at any of the three nitrogen levels applied or in tissue type. However, at the eight-leaf stage the genotype × N interaction was significant ($P < 0.001$) with lower concentrations of $NO_3^-$ in the leaf and root tissue of *acdc1* compared with Sb ($P < 0.001$) at HN and MN. In *tcd1* plants $NO_3^-$ was significantly lower in the sheath tissue ($P < 0.001$) at HN and MN, but was higher under LN. There was also an increase in the $NO_3^-$ in *tcd1* leaf tissue under LN, but otherwise there was no significant difference in $NO_3^-$ levels in leaves and roots of *tcd1* and Sb. At the final harvest, nitrogen treatment still had a significant effect on $NO_3^-$ concentrations for all tissue types ($P < 0.001$), but genotypic effects were only significant in *acdc1* grown at HN ($P < 0.001$), where $NO_3^-$ was lower.

There was a significant difference between the genotypes in the allocation of nitrogen to dhurrin on a whole plant basis, across all harvests (Fig. 3*a*). Such differences were not seen in relation to the allocation of nitrogen to $NO_3^-$ on a whole plant basis. At the three-leaf stage, the allocation of nitrogen to $NO_3^-$ was not significantly different between genotypes (Fig. 3*b*). Following the start of nitrogen treatments there was also no genotypic difference in the allocation of nitrogen to $NO_3^-$ (at the five-leaf stage), although there was less allocated under LN for all lines. Differences between the lines were, however, detected at the eight-leaf stage, with variations in allocation of nitrogen to $NO_3^-$ in *tcd1* compared with Sb, with lower proportions of nitrogen allocated to $NO_3^-$ in *tcd1* at MN and HN and higher allocation at LN (Fig. 3*b*). At the eight- and 10-leaf stages there was a treatment effect, with less allocation to $NO_3^-$ at lower levels of applied nitrogen (Fig. 3*b*).

There were significant differences in how elemental nitrogen was partitioned into dhurrin and $NO_3^-$ in the different lines, tissue types, stages and treatments (Table S3). The proportion of nitrogen allocated to dhurrin was highest in young plants, with 1.80–3.58% in Sb, and with a relatively higher proportion in Sb compared with *acdc1* (0.48–1.83%) (Table S3a). Allocation of nitrogen to dhurrin decreased to below 1% in aboveground tissue as plants aged, but remained high in roots (1–2%; Table S3a). The level of nitrogen applied to the plants also had a significant effect on the proportion of nitrogen allocated to nitrate ($NO_3^-$-N/total N) (Table S3b). Genotype differences in $NO_3^-$-N/N only became significant when plants reached the eight-leaf stage when, in the *tcd1* plants containing no dhurrin, a greater proportion of nitrogen was allocated to $NO_3^-$ in the leaves but less in the sheath, compared with the other two lines. The reverse was true for *acdc1*: even though these plants were also essentially acyanogenic at this leaf stage, less nitrogen was allocated to $NO_3^-$ in the leaves and more in the sheath (Table S3b). In root tissue, Sb and *tcd1* allocated similar amount of nitrogen to $NO_3^-$ whilst *acdc1* allocated significantly less under all nitrogen treatments at the eight- and 10-leaf stages (Table S3b).

Specific leaf nitrogen (SLN) and chlorophyll are both used to indicate photosynthetic potential. Prior to nitrogen treatment there was no significant difference in SLN across the genotypes, but after the treatments were imposed, SLN was higher in *acdc1*

**Table 3. Total elemental nitrogen (N%) of the leaf, sheath and root tissue of *Sorghum bicolor* (Sb) and two cyanide deficient mutants (*acdc1* and *tcd1*)**
Plants were grown at three concentrations of nitrogen: high (HN, 12 mM), medium (MN, 6 mM) and low (LN, 2 mM) and harvested at four leaf stages. Nitrogen treatments were imposed after the first harvest. Values are means ± s.e. ($n=7$ for Sb and *acdc1*, $n=4$ for *tcd1*), except total N for plants at the three-leaf stage where tissue from all individuals was pooled due to small plant size. Total nitrogen (N mg) was calculated by multiplying the concentration by the mass for each tissue. Significance of one-way (three-leaf stage) or two-way ANOVAs (other leaf stages) are presented for each genotype (line, L) and treatment (T): *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$; ns, not significant. Means with the same lower case letter are not significantly different ($P>0.05$; Tukey's pairwise comparison)

| | Sb | | | *acdc1* | | | *tcd1* | | | ANOVA | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | LN | MN | HN | LN | MN | HN | LN | MN | HN | L | T | L × T |
| *Three-leaf stage* | | | | | | | | | | | | |
| Leaf | | 4.7 | | | 4.7 | | | 4.8 | | N/A | | |
| Sheath | | 4.4 | | | 4.3 | | | 3.8 | | N/A | | |
| Root | | 2.6 | | | 2.6 | | | 3.1 | | N/A | | |
| N per plant (mg) | | 2.3 | | | 2.1 | | | 2.4 | | N/A | | |
| *Five-leaf stage* | | | | | | | | | | | | |
| Leaf | 4.2 ± 0.1 | 4.6 ± 0.01 | 4.8 ± 0.03 | 4.7 ± 0.1 | 4.7 ± 0.03 | 4.8 ± 0.1 | 4.0 ± 0.4 | 4.6 ± 0.00 | 4.6 ± 0.1 | ns | *** | ns |
| Sheath | 3.7 ± 0.1 | 4.3 ± 0.04 | 4.7 ± 0.1 | 4.6 ± 0.3 | 4.5 ± 0.1 | 5.2 ± 0.2 | 4.17 ± 0.04 | 4.7 ± 0.1 | 4.7 ± 0.03 | * | *** | ns |
| Root | 2.0 ± 0.1 | 2.0 ± 0.1 | 2.2 ± 0.1 | 2.6 ± 0.6 | 2.1 ± 0.1 | 2.4 ± 0.04 | 1.9 ± 0.4 | 2.4 ± 0.1 | 2.5 ± 0.2 | ns | ns | ns |
| N per plant (mg) | 5.2 ± 0.6 | 7.5 ± 0.3 | 9.1 ± 1.1 | 6.5 ± 1.5 | 9.1 ± 4.3 | 8.3 ± 1.8 | 7.6 ± 2.5 | 9.4 ± 3.9 | 9.3 ± 2.2 | ns | ** | ns |
| *Eight-leaf stage* | | | | | | | | | | | | |
| Leaf | 2.3 ± 0.0 | 3.8 ± 0.1 | 3.9 ± 0.1 | 2.7 ± 0.3 | 3.8 ± 0.1 | 4.3 ± 0.1 | 3.1 ± 0.3 | 4.1 ± 0.1 | 3.9 ± 0.1 | * | *** | ns |
| Sheath | 1.3 ± 0.1 | 3.4 ± 0.1 | 4.0 ± 0.1 | 1.7 ± 0.3 | 3.0 ± 0.4 | 4.1 ± 0.2 | 2.1 ± 0.3 | 3.8 ± 0.2 | 4.2 ± 0.2 | * | *** | ns |
| Root | 0.8 ± 0.0 | 1.5 ± 0.1 | 1.8 ± 0.1 | 1.0 ± 0.1 | 1.5 ± 0.1 | 1.9 ± 0.1 | 1.2 ± 0.1 | 1.6 ± 0.1 | 2.0 ± 0.0 | ** | *** | ns |
| N per plant (mg) | 13 ± 2e | 23 ± 4d | 36 ± 4a | 16 ± 3e | 34 ± 7c | 48 ± 6b | 21 ± 11e | 21 ± 7d | 40 ± 11ab | *** | *** | * |
| *10-leaf stage* | | | | | | | | | | | | |
| Leaf | 1.3 ± 0.09 | 3.1 ± 0.2 | 3.9 ± 0.04 | 1.4 ± 0.2 | 2.8 ± 0.1 | 3.5 ± 0.1 | 1.4 ± 0.2 | 3.0 ± 0.3 | 3.8 ± 0.1 | ns | *** | ns |
| Sheath | 0.6 ± 0.05 | 2.0 ± 0.10 | 3.5 ± 0.03 | 0.5 ± 0.1 | 1.2 ± 0.1 | 2.6 ± 0.1 | 0.6 ± 0.1 | 1.9 ± 0.3 | 3.8 ± 0.5 | ** | *** | ns |
| Root | 0.7 ± 0.03 | 1.1 ± 0.11 | 1.6 ± 0.21 | 0.7 ± 0.1 | 1.1 ± 0.1 | 1.8 ± 0.2 | 0.6 ± 0.1 | 1.2 ± 0.1 | 1.7 ± 0.1 | ns | *** | ns |
| N per plant (mg) | 34 ± 9 | 68 ± 13 | 89 ± 6 | 27 ± 5.96 | 77 ± 33 | 93 ± 18 | 27 ± 10 | 68 ± 29 | 76 ± 13 | ns | *** | ns |

and *tcd1* compared with Sb at the eight- and 10–leaf stages, particularly under LN treatments (Table S2). Few significant differences were detected in the concentration of chlorophyll or carotenoids until the later growth stages, when all lines grown at LN had significantly less chlorophyll (Table S4).

### *Transcript expression levels of key genes governing dhurrin synthesis and nitrogen turnover*

In order to determine whether dhurrin concentration is transcriptionally regulated, the expression of key genes in the biosynthetic pathway (*CYP79A1*), turnover pathways (*NIT4B2*) and general nitrogen metabolism (*NR1*, *nitrate reductase* and *GS2*, *glutamine synthetase 2*) were measured. The expression of *CYP79A1* (the gene encoding the synthesis of the first committed enzyme in the biosynthetic pathway for dhurrin) was very low in both leaf and root tissue of all genotypes and at all leaf stages, even in very young plants when dhurrin concentrations were uniformly high (Fig. 4*a, e*). However, at the eight-leaf stage *CYP79A1* expression was significantly higher in the leaf tissue of Sb plants. *NIT4B2*, which is involved in both the detoxification and endogenous turnover pathways, showed higher expression levels than *CYP79A1*. In leaf tissue, *NIT4B2* transcript levels were significantly higher in *acdc1* compared with Sb and *tcd1* plants, up to and including the eight-leaf stage (Fig. 4*b*). However, by the 10-leaf stage there were no significant differences in *NIT4B2* expression within genotypes or treatments (Fig. 4*b*). *NIT4B2* expression did not differ significantly in the roots for genotype or within treatment although, again, the trend was towards higher expression at LN, suggesting an increase in the alternative turnover pathway (Fig. 4*f*).

Nitrate reductase (NR) catalyses the reduction of nitrate to nitrite. Here, we detected high levels of expression of the *NR1* gene in the leaves (compared with all other genes) and in the roots (in comparison to all genes except *GS2*). There were significant differences in *NR1* expression among treatments, genotypes and tissue-types (Fig. 4*c, g*). At the five-leaf stage, expression in the leaf tissue was significantly higher for all three genotypes under HN (12 mM) compared with the other two nitrogen treatments. Transcript levels then decreased with age, except for *tcd1* which remained elevated at LN (Fig. 4*c*). In the root tissue, *NR1* transcript levels were significantly lower in *acdc1* compared with Sb at the five-leaf stage, but as plants aged there was no significant difference between genotypes or due to nitrogen treatment until the 10-leaf stage when there was a significant increase in expression under LN (Fig. 4*g*).

Glutamine synthetase 2 (GS2) is involved in the recycling of $NH_3/NH_4$ and glutamate to glutamine, which is important for nitrogen use efficiency (Kumagai *et al*. 2011; Xu *et al*. 2012). There was a significant difference in expression of *GS2* in leaf and root tissue, with much higher expression levels in the root (Fig. 4*d, h*). We noted that in the leaf tissue *tcd1* plants had higher expression under LN than both other lines at the five- and eight-leaf stages, possibly to improve access to nitrogen from sources other than dhurrin. By the 10-leaf stage there was a significant difference in *GS2* expression at HN for all genotypes, but *acdc1* also had significantly higher expression at LN. In the root tissue, there was little difference in *GS2* transcript levels until the final

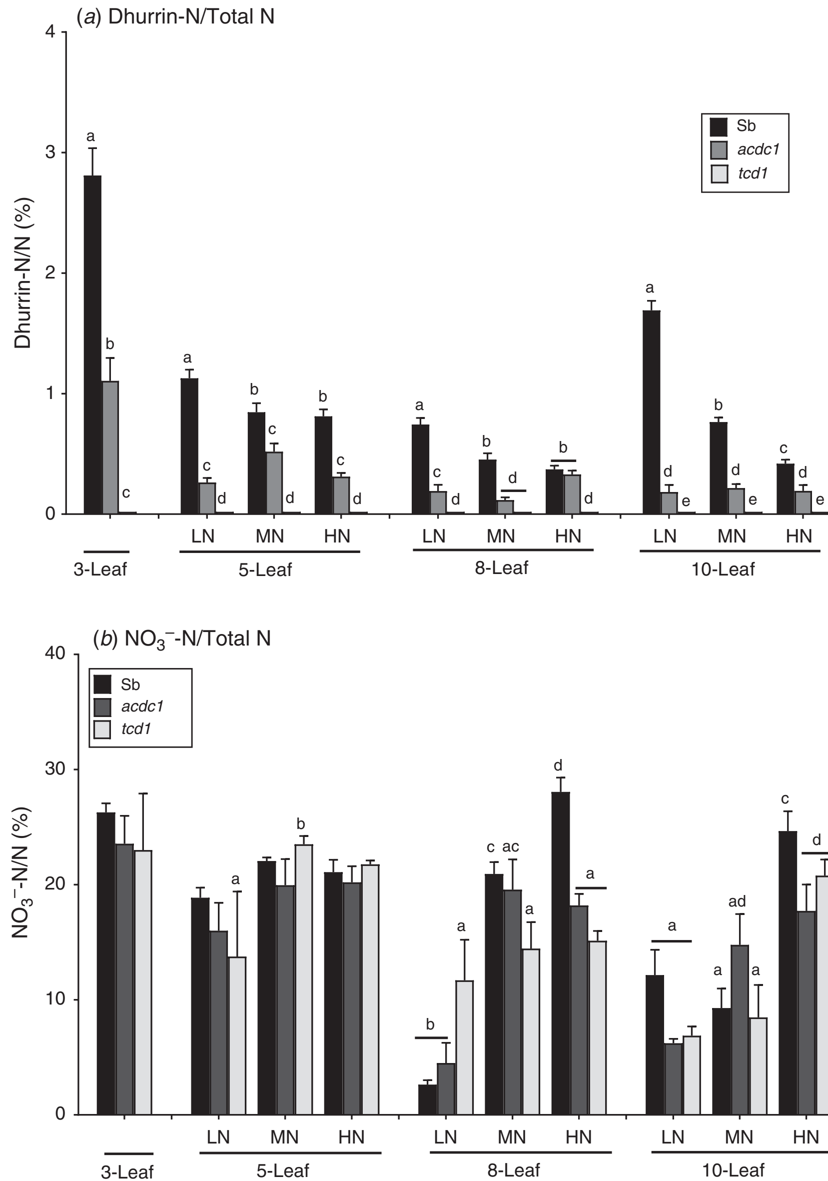


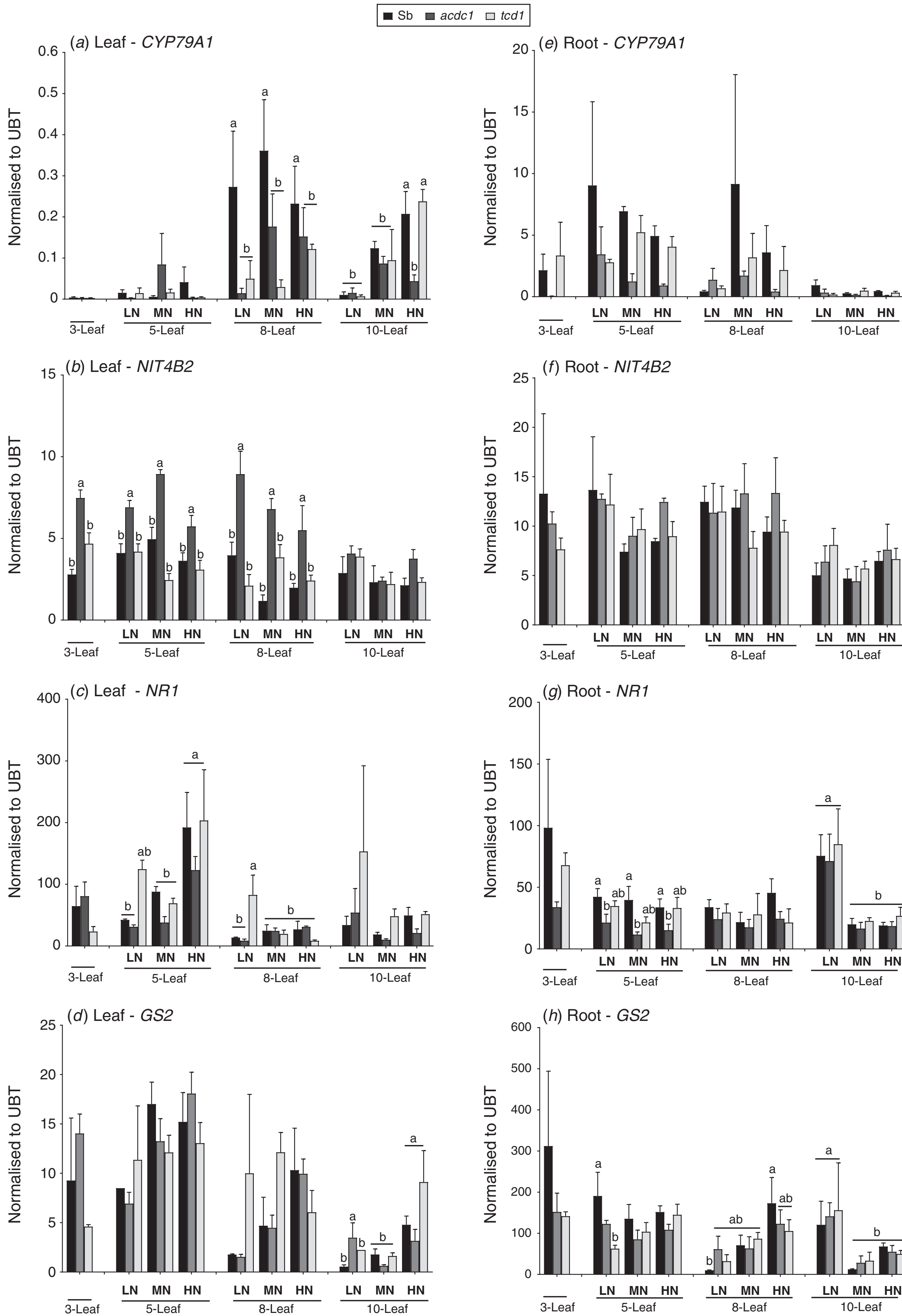

**Fig. 3.** Proportion of nitrogen allocated to (*a*) dhurrin, and (*b*) nitrate on a whole plant basis in wild type *Sorghum bicolor* (Sb) and mutants that are either totally acyanogenic (*tcd1*) or acyanogenic as adults (*acdc1*). Plants grown at three concentrations of nitrogen (2, 6 and 12 mM; low nitrogen (LN), medium nitrogen (MN) and high nitrogen (HN) respectively) and harvested at the three-, five-, eight- and 10-leaf stages. Values are means ± s.e. ($n = 7$ for Sb and *acdc1*, $n = 4$ for *tcd1*). Within harvests, columns with different letters are significantly different at $P < 0.05$, analysed using ANOVA and Tukey's test. A more detailed breakdown of allocation to particular tissues (leaves, sheath and roots) can be found in Table S3, available as Supplementary Material to this paper.

harvest (10-leaf stage) when expression was significantly higher under LN in all genotypes (Fig. 4*h*).

## Discussion

### *Investment in growth and defence are dependent on both source and sink*

Cyanogenic glucosides like dhurrin are mostly constitutively produced but the biosynthetic pathway catalysing their formation is organised within a dynamic metabolon enabling rapid adjustment to abiotic and biotic stresses (Laursen *et al.* 2016; Bassard *et al.* 2017). As part of a two component defence system, cyanogenic glucosides exert their defence function by their ability to liberate toxic hydrogen cyanide as a response to tissue disruption, e.g. by a chewing insect (Gleadow and Møller 2014). Given the energetic costs and resources required for their synthesis, it is usually assumed that there must be a trade-off with growth, such that plants with higher concentrations would be


Sb
acdc1
tcd1
(a) Leaf - CYP79A1
(b) Leaf - NIT4B2
(c) Leaf - NR1
(d) Leaf - GS2
(e) Root - CYP79A1
(f) Root - NIT4B2
(g) Root - NR1
(h) Root - GS2
Normalised to UBT
LN
MN
HN
3-Leaf
5-Leaf
8-Leaf
10-Leaf

smaller. However, such trade-offs have been very difficult to demonstrate. Since *tcd1* does not synthesise dhurrin, we hypothesised that these plants would have higher growth rates, especially when nitrogen was limiting for growth. We also had the opportunity to track the cost of changes in allocation over time with the *acdc1* plants, such that they would only display a growth benefit after they transitioned into the acyanogenic form. We found that *acdc1* did grow significantly faster at later stages of development, but the totally acyanogenic *tcd1* grew more slowly than the wild-type controls, at least initially. These results support the hypothesis that in early development dhurrin may be required for effective management of plant nitrogen, but that in older plants, synthesis comes at a cost and there is a trade-off between growth and defence.

Overall, the level of applied nitrogen was the primary driver of the observed differences in growth between the genotypes, with faster plant growth and rates of development seen when levels of nitrogen were high. Jenrich *et al*. (2007) proposed an alternative dhurrin turnover pathway, generating ammonia without the release of toxic HCN. Detailed analysis of the synthesis and turnover of CNglcs in several species by Pičmanová *et al*. (2015) showed that CNglcs co-occur with several structural derivatives, including amides and acids. Based on these results, Pičmanová *et al*. (2015) modified and expanded the alternative pathway, suggesting that it runs in parallel with the detoxification pathway providing a ready source of reduced nitrogen. The presence of these structural derivatives suggests that the turnover pathway is dynamic and that these compounds may be a source of carbon and nitrogen for use in primary metabolism during early plant development. This may explain why *tcd1* initially grows slowly. It may be possible to achieve wild-type growth rates in *tcd1* with high levels of nitrogen supply early in development, earlier than the stages we tested, although it would need to be supplied in a reduced form and not nitrate as used here, to conserve energy that would otherwise be used for growth.

The allocation of nitrogen to dhurrin (dhurrin-N/N%) was highest in the very young Sb plants, with ~3% allocation on a whole plant basis (Fig. 3*a*), ranging from 2–4% depending on the tissue type (Table S3). Allocation then decreased with age in above ground tissue, in line with the known decrease in HCNp as sorghum matures (Miller *et al*. 2014). We note that allocation of nitrogen to dhurrin in root tissue remained relatively constant within each treatment, similar to results from a previous study of drought stressed sorghum (O'Donnell *et al*. 2013). Allocation was significantly less in the above ground tissue for *acdc1* plants, but similar to Sb in root tissue (Table S3). Taken together, these studies indicate that the allocation may be regulated independently in roots and shoots. In the older plants, nitrogen allocation to dhurrin was higher under limiting nitrogen, possibly due to the longer time for the plants grown at low nitrogen to reach the 10-leaf stage, resulting in a higher leaf dry weight. This is in agreement with Gleadow and Woodrow (2000*a*, 2000*b*), who showed that for eucalypts, when the plants were nitrogen-limited, they continued to synthesise a base level of cyanogenic glucosides, but when nitrogen was available at luxury levels, the relative allocation of nitrogen to HCN was lower, even though the overall cyanogenic glucoside concentration was higher.

Our other hypothesis was that since acyanogenic plants were unable to store nitrogen as dhurrin, they would divert 'excess' nitrogen to nitrate. The total amount of nitrate, either as a proportion of plant nitrogen per unit mass, or on a whole-plant basis was highly dependent on nitrogen supply and plant age, confirming the role of nitrate as a storage compound in sorghum (Busk and Møller 2002; Gleadow *et al*. 2016). During early growth there was little variation between the three genotypes in how nitrogen was partitioned in any tissue type. However, at the eight-leaf stage allocation to nitrate was higher in the leaf tissue of *tcd1* for all treatments, compared with *acdc1*, but in the sheath tissue *tcd1* plants allocated significantly lower amounts of nitrogen to nitrate. This is unusual, as typically sorghum stores nitrate in the sheath tissue when nitrogen supply is high (White *et al*. 2016). The observation that the intermediate treatments give the strongest response may be because at low nitrogen, when nitrogen is limiting, any diversion of resources impacts plant growth.

### Molecular regulation of dhurrin and N turnover is complex

To elucidate the underlying molecular mechanisms, we measured transcript levels of key genes involved in dhurrin synthesis (*CYP79A1*) and turnover (*NIT4B2*) (Gleadow and Møller 2014), and two genes involved in nitrogen assimilation (*nitrate reductase*; *NR1* and *glutamine synthetase*; *GS2*) (Fig. 1; Xu *et al*. 2012; Gelli *et al*. 2014). The P450 cytochrome oxidase CYP79A1 catalyses the first step in the biosynthesis pathway (Koch *et al*. 1995), whereas the nitrilase, NIT4B2, has been shown to be involved in the alternative turnover pathway of dhurrin, forming ammonia (Jenrich *et al*. 2007). Nitrate is ultimately converted to ammonium by sequential action of nitrate reductase (NR) and then by nitrite reductase (NiR); and the ammonium further assimilated into amino acids via the GS/glutamate synthase (GOGAT) cycle (Xu *et al*. 2012).

Dhurrin synthesis has been shown to be transcriptionally regulated in seedlings (Busk and Møller 2002) and the developing grain (Nielsen *et al*. 2016). Here, *CYP79A1* transcript levels were very low in the leaf and root tissue of plants at the three- and five-leaf stage, even when HCNp was quite high. A similar disjunction between product and gene expression has been observed for glucosinolates in *Arabidopsis*, leading the authors of that work to postulate that the defence response may be based on a network of interactions rather than a simple correlation between gene expression and glucosinolate production (Rasmann *et al*. 2015). This may also be the case in sorghum. In very young sorghum seedlings, the very high expression of *CYP79A1* observed by Busk and Møller

**Fig. 4.** Expression levels in leaves and roots of key genes that are involved in dhurrin synthesis, the alternative dhurrin turnover pathway, and nitrogen assimilation for three Sorghum genotypes (wild type, Sb; totally cyanide deficient, *tcd1*; adult cyanide deficient class 1 mutants, *acdc1*). Transcripts were normalised relative to ubiquitin expression (UBT). *CYP79A1* governs the first step in the synthesis of dhurrin; *NIT4B2* encodes the enzyme that catalyses the conversion of β-cyanoalanine to asparagine and $NH_3$; *nitrate reductase*, *NR* and *glutamine synthetase*, *GS* encode key enzymes in the nitrogen reduction pathway (see Fig. 1). Values are means ± s.e. ($n = 3$ for all genotypes). Columns marked with different letters are significantly different at $P < 0.05$, analysed using ANOVA and Tukey's test.

(2002) could lead the system to saturate, obviating the need for more synthesis. This is potentially regulated through a feedback loop, similar to the system observed for ascorbate synthesis (Laing *et al*. 2015).

The expression of *NIT4B2* was higher in the *acdc1* mutant than the other two lines, suggesting an increased activity and higher rates of turnover. This could potentially mobilise additional nitrogen resources leading to improved growth, particularly at low nitrogen treatments, and is consistent with our observations. This is supported by the results from the analysis of the developing sorghum grain, which showed that although the mature grain is acyanogenic, dhurrin levels are quite high in the immature grains (Nielsen *et al*. 2016). Concomitant with this decrease in dhurrin is an increase in *NIT4A* and *NIT4B2* expression, indicating that the endogenous turnover pathway operates in the seed to provide an alternative nitrogen source for seed storage protein production (Nielsen *et al*. 2016).

Nitrate is a major source of nitrogen for plant growth, but it must be reduced to ammonium before it can be used for amino acid or protein synthesis. NR activity is induced by nitrate, but there is a limit to how much $NO_3^-$ can be reduced, either by the availability of $NADPH^+$ from photosynthesis or from storage. NR activity is also inhibited by cyanide (Siegień and Bogatek 2006; Yu and Zhang 2012). Consistent with this, we observed higher levels of *NR* transcripts in the leaves, compared with the roots, for all three genotypes, particularly during early plant development when the plants are grown at HN. *NR* expression is known to be induced by high nitrate availability (Stitt and Krapp 1999; Balotf *et al*. 2016). This was true for the leaves of *acdc1* and Sb plants, but in *tcd1* plants, *NR1* transcript levels were higher in leaves of plants from the LN treatment group. *GS2* transcript levels were also higher in *tcd1* leaf tissue. The combination of high *NR1* and *GS2* transcript levels under LN in *tcd1* plants suggests that this genotype is maximising its use of nitrate when availability is low. This is not necessarily a trade-off but an adaptation for higher nitrogen use efficiency and could be an added benefit of this non-toxic line. We also observed higher *NR1* transcript levels in the root tissue of all genotypes grown under low nitrogen levels.

### *Iterative regulation of plant defence and growth: implications for crop improvement*

Measurements of growth/defence trade-offs are typically made using natural variation within or among populations for a particular defence metabolite. Here we used a null mutant and another line with a mutation that appears to be developmentally regulated. We observed clear differences in the growth and allocation of nitrogen between these genotypes, with the *acdc1* mutant generally performing better. Our analysis indicates that this is a complex relationship. One reason trade-offs are difficult to measure is that comparisons are often made in plants of the same chronological age, and yet partitioning of resources is both age (Barton and Koricheva 2010) and resource dependent (Coley 1988). An additional confounding variable is that plants can use secondary metabolites for primary purposes, not just defence; thus, there is not necessarily a clear-cut trade-off between them (Coley 1988; Herms and Mattson 1992; Neilson *et al*. 2013; Gleadow and Møller 2014). Rather than measuring trade-offs, we conclude the metabolism is more of a web with iterative control, regulated at the molecular level through synthesis, turnover and storage.

In order to increase crop productivity it is essential to understand the impact of source and sink on resource allocation, and the impact of genetically manipulating them (White *et al*. 2016). We initially hypothesised that the *tcd1* mutant would have improved growth as it would have access to more available nitrogen, but this did not seem to be the case. The results suggest that dhurrin is required in early plant development. Cassava with significantly reduced levels of CNglcs has been generated using RNAi. Analysis of these plants identified distinct phenotypic characteristics, including the requirement for additional nitrogen to ensure root establishment (Jørgensen *et al*. 2005). This also suggests that CNglcs have a function as an alternative nitrogen source. There did appear to be an advantage in not synthesising dhurrin when plants were older, suggesting a potential trade-off between growth and defence. These differences in response to the presence/absence of CNglcs at different stages of plant development may be due to the fact that dhurrin has been proposed to have multiple and important roles in the primary metabolism in nitrogen turnover and metabolism (Gleadow and Møller 2014; Pičmanová *et al*. 2015) as well as in mitigating the effect of reactive oxygen species resulting from pathogen attack (Møller 2010). The variations in HCNp in different tissue types, and under differing nitrogen regimes for the three genotypes, supports the hypothesis that synthesis and metabolism of CNglcs is highly dynamic and not merely involved in defence (Pičmanová *et al*. 2015).

## Conflicts of interest

The authors declare no conflicts of interest.

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