STUDIES

Providing carbon skeletons to sustain amide synthesis in roots underlines the suitability of Brachypodium distachyon for the study of ammonium stress in cereals

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Abstract

Plants mainly acquire N from the soil in the form of nitrate (NO\textsubscript{3}\textsuperscript{−}) or ammonium (NH\textsubscript{4}\textsuperscript{+}). Ammonium-based nutrition is gaining interest because it helps to avoid the environmental pollution associated with nitrate fertilization. However, in general, plants prefer NO\textsubscript{3}\textsuperscript{−} and indeed, when growing only with NH\textsubscript{4}\textsuperscript{+} they can encounter so-called ammonium stress. Since Brachypodium distachyon is a useful model species for the study of monocot physiology and genetics, we chose it to characterize performance under ammonium nutrition. Brachypodium distachyon Bd21 plants were grown hydroponically in 1 or 2.5 mM NO\textsubscript{3}\textsuperscript{−} or NH\textsubscript{4}\textsuperscript{+}. Nitrogen and carbon metabolism associated with NH\textsubscript{4}\textsuperscript{+} assimilation was evaluated in terms of tissue contents of NO\textsubscript{3}−, NH\textsubscript{4}+, K, Mg, Ca, amino acids and organic acids together with tricarboxylic acid (TCA) cycle and NH\textsubscript{4}+ assimilating enzyme activities and RNA transcript levels. The roots behaved as a physiological barrier preventing NH\textsubscript{4}+ translocation to aerial parts, as indicated by a sizeable accumulation of NH\textsubscript{4}+, Asn and Gln in the roots. A continuing high NH\textsubscript{4}+ assimilation rate was made possible by a tuning of the TCA cycle and its associated anaplerotic pathways to match 2-oxoglutarate and oxaloacetate demand for Gln and Asn synthesis. These results show B. distachyon to be a highly suitable tool for the study of the physiological, molecular and genetic basis of ammonium nutrition in cereals.

Keywords: Ammonium assimilation; Asn; carbon metabolism; Gln; monocots; nitrate; nitrogen metabolism; root; TCA cycle.

Introduction

Over the last decade, Brachypodium distachyon has gained attention as model plant for C3 grasses. Phylogenetically, it lies between rice and wheat, with a high degree of sequence similarity with wheat, and high degree of synteny with most grasses (Brutnell et al. 2015). Given B. distachyon is not domesticated, it shows great intra-species diversity; its pan-genome containing nearly twice the number of genes found in any individual genome (Gordon et al. 2017).

Although many aspects of Brachypodium development and responses to biotic and abiotic stresses have been studied, little has been published concerning Brachypodium nitrogen (N) signalling and metabolism (Ingram et al. 2012; Poiré et al. 2014; Barhoumi 2017) and, to our knowledge, no report is available on how B. distachyon deals with different N sources. This point is crucial since N is the major mineral nutrient demanded by plants and its availability is yield-limiting in many agronomic soils (Xu et al. 2012). Plants take up N mainly in form of ammonium (NH\textsubscript{4}/NH\textsubscript{4}+) and nitrate (NO\textsubscript{3}−). Nitrate is usually the preferred source but is a source of pollution because anions are readily lost through leaching. Besides, nitrous oxide (N\textsubscript{2}O), one
of the strongest greenhouse gases, is emitted during bacterial denitrification (Huérfano et al. 2015). Ammonium salts, when combined with nitrification inhibitors, are more stable in the soil and have been proved useful in mitigating some of the unwanted effects of nitrate fertilization (Huérfano et al. 2015). Moreover, ammonium nutrition can sometimes confer positive effects on plant performance, for example by increasing sorghum and rice tolerance to osmotic stress (Gao et al. 2010, Miranda et al. 2016). It has also been suggested that ammonium nutrition may improve the response of some species to high concentrations of atmospheric CO2 (Bloom et al. 2010). In addition, a frequent characteristic associated with ammonium nutrition is an enrichment with N-containing compounds (Marino et al. 2016; Coleto et al. 2017). However, ammonium nutrition is also known to decrease plant growth. This is the main symptom of ammonium stress, the so-called ‘ammonium syndrome’ (Liu and Von Wirén 2017). The energetic cost associated with maintaining cytosolic NH3/NH4+ homeostasis, mainly by pumping NH3/NH4+ out of the cytosol and by increasing NH3 assimilation, is considered to be one of the major causes of biomass reduction (Britto and Kronzucker 2002; Esteban et al. 2016). If the concentration of NH3/NH4+ exceeds the capacity for efflux and assimilation, NH3+ is, in most species, preferentially accumulated in root cells to avoid damaging the photosynthetic apparatus (Esteban et al. 2016). Overall, the study of the metabolic adaptation to ammonium stress is crucial to increase plant N use efficiency while reducing N losses associated with nitrate fertilization.

Ammonium is mainly assimilated via the glutamine synthetase/glutamate synthase (GS/GOGAT) cycle. To sustain GS/GOGAT activity, the tricarboxylic acid (TCA) cycle and its associated routes regulate the continuous supply of carbon skeletons. Indeed, proper management of carbon supply has been shown to be essential for ammonium tolerance (Roosta and Schjoerring 2008; Vega-Mas et al. 2015). Although controlling NH3/NH4+ entry/efflux and its assimilation is crucial for NH3+ homeostasis, ammonium stress is also related to other processes such as pH control, ion imbalance and nitrate signalling (Liu and Von Wirén 2017). The study of the co-ordination and regulation of all these mechanisms is essential to understand fully how they determine the extent of tolerance/sensitivity to ammonium nutrition in a given species or genotype. For instance, there is considerable inter- and intraspecific variability in the extent of ammonium stress amongst grass species such as maize (Schortmeyer et al. 1997), rice (Chen et al. 2013) and wheat (Wang et al. 2016a).

In this work, we undertook a comprehensive physiological and metabolic characterization of B. distachyon (reference genotype Bd21) grown with exclusive access to NH3+ or NO3− as N source. We focused on leaf and root carbon metabolism and on nitrogen assimilatory pathways.

Methods

Growth conditions and experimental design

Brachypodium distachyon Bd21 seeds were sterilized in 100 % ethanol for 1 min, rinsed three times with deionized water and incubated in deionized water for 2 h. Then, seeds were placed in trays filled with a perlite-vermiculite (1:1) mixture moistened with deionized water. After 4 days of stratification at 4 °C in the dark, the trays were transferred to a growth chamber (60/70 % of relative humidity, 23 °C day (14 h) with a light intensity of 350 μmol m−2 s−1 and 18 °C night (10 h)). Eleven days after sowing, seedlings were transferred to 4.5-L hydroponic tanks (10 plants per tank). The nutrient solution contained 1.15 mM K2HPO4, 0.85 mM MgSO4, 0.7 m mM CaSO4, 2.68 mM KCl, 0.5 mM CaCO3, 0.07 mM NaFeEDTA, 16.5 μM NaNO3, 3.7 μM FeCl3, 3.5 μM ZnSO4, 16.2 μM H3BO3, 0.47 μM MnSO4, 0.12 μM CuSO4, 0.21 μM AlCl3, 0.126 μM NiCl2 and 0.06 μM KI, pH 6.8. The nitrogen source was (NH4)2SO4 for ammonium-fed plants and Ca(NO3)2 for nitrate-fed ones. Each N source was supplied at 1 or 2.5 mM of total N. To compare both N sources within each concentration, NO3−-fed plants were supplied with CaSO4 to match the SO4−2 supplied with the NH4+. The pH of the solution was checked every 2 days and the nutrient solution replaced every 4 days. Four tanks were set up per treatment; thus, a total of 40 plants were grown per condition. Twenty-four days after transfer to hydroponic conditions, plants were harvested. Shoots and roots were separated and individually weighed. For metabolic measurements, plants grown in the same tank were pooled and immediately frozen in liquid nitrogen, homogenized in a Tissue Lyser (Retsch MM 400) and stored at −80 °C until use.

Element and metabolite determination

Nitrogen and carbon content was determined with an elemental analyser Flash EA1112 (Thermo Fisher Scientific Inc., Waltham, MA, USA). Chlorophyll was extracted in 80 % aqueous acetone and quantified spectrophotometrically (Arnon 1949). Ammonium was extracted from 50 mg of frozen leaf and root powder as described in Sarasketa et al. (2014) and quantified following the phenol hypochlorite method. Nitrate was determined as described in Tschoep et al. (2009). Inorganic elements were extracted from 10 mg of lyophilized leaf and root with HNO3 and microwave-assisted digestion (Mars5, Vertex, Spain). Quantification was performed using an optical emission spectrophotometer with inductively coupled plasma ICP-OES (Horiba Jobin Yvon, Activa). Amino acids were determined by capillary electrophoresis (PA-800, Beckman Coulter Inc., USA) coupled with laser-induced fluorescence detection (argon laser at 488 nm) as previously described (Orcaray et al. 2010). Organic acids were extracted from 150 and 200 mg of frozen leaf and root, respectively, in 1.8 mL of methanol:water:chloroform (4:4:10). After centrifugation, the upper layer was recovered, vacuum dried and the pellet suspended in 1 mL of ultrapure water that was finally filtered through a 0.22-μm PES filter. Separation and quantification were performed by ion chromatography (Dionex ICS-5000, Thermo Scientific).

Protein content and enzyme activities determination

Protein was extracted from 100 mg of frozen leaf and root powder with 1 mL extraction buffer as described in Sarasketa et al. (2016). Protein was quantified using a Bradford base dye-binding assay (Bio-Rad, Hercules, CA, USA) with bovine serum albumin as a standard. Enzyme activities were determined with a 96-well plate spectrophotometer (BioTek Instruments). Glutamine synthetase (GS), NADH glutamine 2-oxoglutarate aminotransferase (GOGAT), NAD(H)- and NADP(H)-dependent glutamate dehydrogenase (GDH and NADP-GDH, respectively) in its aminating sense, NADP-dependent isoctrate dehydrogenase (ICDH), NAD- and NADP-dependent malic enzymes (ME), malate dehydrogenase (MDH) and phosphoenolpyruvate carboxylase (PEPC) were assayed as described in Coleto et al. (2017). Aspartate aminotransferase (AAT) was measured as in Gordon and Kessler (1990).

RNA extraction and gene expression analysis

RNA extraction was from 25 mg of frozen leaf or root powder with the Nucleospin RNA plant kit (Macherey-Nagel) that includes DNase treatment. One microgram of RNA was retrotranscribed.
into cDNA (PrimeScript™ RT; Takara Bio Inc.) and gene expression was determined from 2 µL of cDNA diluted 1:10 in a 15 µL reaction volume using SYBR Premix ExTaq™ (Takara Bio Inc.) in a Step One Plus Real Time PCR System (Applied Biosystems). The PCR programme was 95 °C for 5 min followed by 40 cycles of 94 °C for 15 s and 60 °C for 1 min and a melting curve (40–95 °C with one fluorescence read every 0.3 °C). ACT3 and SamDC were used as housekeeping genes to normalize gene expression. Absence of genomic DNA contamination was checked in all RNA samples. In Supporting Information—Table S1, the primers used and their efficiency, calculated with serial cDNA dilutions, are described.

**Phylogenetic analysis**

Phylogenetic analysis was done using the programmes included in the Phylogeny.fr tool (Dereeper et al. 2008) as follows. Coding sequences multiple alignments were conducted with the Muscle algorithm and refined using G blocks. Phylogeny analysis was done using the bootstrapping procedure with the PhyML software. Finally, TreeDyn was used to visualize the tree.

**Statistical analysis**

All the results presented are given as means with standard errors. Data were analysed with SPSS 17.0 (Chicago, IL, USA). Normality and homogeneity of variance were analysed by Kolmogorov-Smirnov and Levene’s tests. The significance of the results was assessed using independent samples t-test or two-way ANOVA.

**Results**

We compared the performance of B. distachyon Bd21 growing under the exclusive supply of NH₄⁺ as N source with that of plants given an exclusive supply of NO₃⁻ as the control condition. Concentrations of 1 or 2.5 mM of each N source were given. Statistical analyses indicated that biomass was affected by the N source, its concentration and there was also a significant statistical interaction (Fig. 1A). Notably, when plants were grown in 1 mM ammonium growth by roots or shoots was closely similar to that by plants in 1 mM nitrate. When NH₄⁺ was increased to 2.5 mM, plants showed symptoms of ammonium stress, i.e. a lower biomass compared to plants in 2.5 mM nitrate (Fig. 1A). Chlorophyll content was not affected by the treatment (Fig. 1B). Therefore, 1 mM ammonium represented a non-toxic supply while 2.5 mM was toxic.

To analyse N metabolism we determined contents of NH₄⁺, NO₃⁻, protein, total N, individual amino acids and the activity of enzymes related to N metabolism: GS, GOGAT, AAT and glutamate dehydrogenase (GDH) in both leaf and root (Figs 2 and 3). The accumulation of ammonium (NH₄⁺) is the most common metabolic marker of ammonium stress. In agreement with biomass and chlorophyll content, NH₄⁺ content did not accumulate in plants fed with 1 mM ammonium (Figs 2 and 3). However, with 2.5 mM supply, root tissue accumulated ca. 25 times more NH₄⁺ compared to equivalent nitrate nutrition (Fig. 3) whereas in leaf tissue, little NH₄⁺ accumulated (Fig. 2). As expected, NO₃⁻ content was affected by the N source with NO₃⁻ levels being much higher for plants grown in nitrate. Indeed, in plants fed with ammonium NO₃⁻ content was so low it approached the limit of detection of the methodology used [see Supporting Information—Fig. S1].

Asn and Gln were the most abundant amino acids in root and leaf under both NO₃⁻ and NH₄⁺, with Glu also being abundant in plants fed with nitrate (Figs 2 and 3; see Supporting Information—Tables S2 and S3). At 1 mM, differences between NO₃⁻- and NH₄⁺-fed plants were small. Exceptions were Asn and Phe contents which were markedly higher in roots of plants grown with ammonium (Fig. 3; see Supporting Information—Table S3). With 2.5 mM NH₄⁺ supply a large accumulation of NH₄⁺ in the roots was associated with much increased levels of amino acids [see Supporting Information—Table S3], mainly in form of Asn and Gln where contents increased ca. 17 and 19 times, respectively (Fig. 3). Overall, total amino acids content was ca. 8 times higher compared to nitrate nutrition (Fig. 3).

A substantial N source effect was observed in root GDH (NADH- and NADPH-dependent) and GS enzyme activities (Fig. 3; see Supporting Information—Fig. S2A) under both 1 and 2.5 mM. Glutamate dehydrogenase showed high activity in the root of plants fed with ammonium, while the contrary was the case for GS activity (Fig. 3). The stimulation of N assimilation in plants fed with ammonium was also evident in terms of Gln/Glu and Asn/Asp ratios (see Supporting Information—Fig. S3). Importantly, the increase in Gln/Glu and Asn/Asp ratios in the leaves of plants grown with 2.5 mM of ammonium was not only due to Gln and Asn increase but also to a decrease in Glu and Asp (Fig. 2).

To evaluate the regulation of ammonium assimilation enzymes, we looked for genes encoding GS, GDH and AS in B. distachyon Bd21. We carried out BLAST analysis in GenBank (https://www.ncbi.nlm.nih.gov/genbank/) and Phytozome.
(https://phytozome.jgi.doe.gov/) databases, using available sequences for Oryza sativa, Triticum aestivum and Arabidopsis thaliana GS, GDH and ASN genes as queries. We found complete sequences for four genes encoding for GS (three GS1 and one GS2), two genes encoding for GDH and one for NADP-GDH and three genes encoding for AS [see Supporting Information—Table S1]. We performed a phylogenetic analysis for the three families including sequences for A. thaliana, T. aestivum, O. sativa, Hordeum vulgare and Aegilops tauschii [see Supporting Information—Figs S4–S6]. In general, B. distachyon genes lie between Triticeae and rice genes. Arabidopsis genes were more difficult to position [see Supporting Information—Figs S4–S6]. We analysed gene expression by qPCR in leaf and root tissue of plants grown for 24 days with 2.5 mM nitrate or ammonium supply. In roots, expression of BdGLN1;3 (Fig. 4A), BdGDH2 (Fig. 4B), BdASN1 and BdASN3 (Fig. 4C) was higher in plants under
ammonium nutrition. In contrast, the expression of BdGLN2 was higher in roots of plants fed with nitrate (Fig. 4A). In leaves, only BdASN3 expression significantly increased in plants grown with ammonium (Fig. 4C).

The TCA cycle and its associated pathways are essential to supply carbon skeletons for N assimilation, especially under ammonium nutrition. Thus, we determined the contents of relevant organic acids and the activity of a number of TCA-related enzymes (Figs 5 and 6; see Supporting Information—Fig. S2B). In the leaf, no differences were observed in the activity of TCA-related enzymes, except for NAD-ME (Fig. 5; see Supporting Information—Fig. S2B). Nevertheless, ammonium nutrition provoked a remarkable decrease in the content of each organic acid (Fig. 5). In roots, we observed higher activity of NADP-dependent isocitrate dehydrogenase (ICDH), NADP-dependent malic and phosphoenolpyruvate

Figure 3. Ammonium assimilation in B. distachyon root tissue. Plants were grown with nitrate or ammonium as N source at 1 or 2.5 mM for 24 days. Ammonium, total amino acids, Asn, Gln, Glu, Asp and 2-OG content are expressed µmol g⁻¹ FW. For enzyme activities, GOGAT, GDH and AAT are expressed as µmol NADH g⁻¹ FW h⁻¹ and GS as µmol γ-GHm g⁻¹ FW h⁻¹. Protein content is expressed as mg BSA g⁻¹ FW and total N content as % dry wt. Significant differences according to two-way ANOVA are indicated by S for N source effects, C for N concentration effects and S × C for interactions (P < 0.05).
Carboxylase (PEPC) enzymes in plants grown with ammonium supply (Fig. 6; see Supporting Information—Fig. S2B). Access to nitrate or ammonium as N source brought about notable alterations in the content and distribution of organic acids in the root. Changes were mostly observed in 2.5 mM ammonium nutrition, where 3-PGA, PEP, 2-OG and malate contents were lower in plants grown with nitrate (Fig. 6). On the contrary, a significant source × concentration interaction was observed for pyruvate + oxaloacetate (Pyr + OAA) and citrate, whose levels were higher in plants fed with 2.5 mM ammonium compared to those fed with nitrate (Fig. 6). Finally, since ammonium has been shown by others to provoke an imbalance of essential cations, we assessed whether this was also the case for B. distachyon. In roots of plants grown with 2.5 mM of ammonium, Ca, Mg, and K contents were diminished compared to their nitrate counterparts [see Supporting Information—Table S4]. In leaves, K also decreased significantly in plants fed with ammonium [see Supporting Information—Table S4].

Discussion

Ammonium stress is considered universal in most if not all biological systems (Britto and Kronzucker 2002). However, great variability in ammonium-use efficiency has been reported (Britto and Kronzucker 2013; Sarasketa et al. 2014) and there are plant species and genotypes which display high tolerance of ammonium. Indeed, the Poaceae is considered to be relatively tolerant. For instance, ryegrass (Lolium perenne), wheat and notably rice are able to manage adequately the presence of high NH$_4^+$ concentrations in the external medium (Britto et al. 2001; Belastegui-Macadam et al. 2007; Setién et al. 2013). Additionally, intraspecific variability in ammonium toxicity has also been reported amongst different species, including cereals (Schortemeyer et al. 1997; Chen et al. 2013; Wang et al. 2016a).

Brachypodium distachyon has emerged as an excellent model species to study different aspects of development in C3 grasses and responses to a number of environmental constraints (Brutnell et al. 2015). However, studies of nitrogen metabolism remain scarce. Accordingly, we characterized the performance of Bd21, the reference accession for B. distachyon, supplied with nitrate or ammonium as N source with a special focus on interactions between carbon and nitrogen metabolisms.

Brachypodium distachyon Bd21 appeared to be moderately tolerant to ammonium. Indeed, in hydroponic culture it grew equally in 1 mM N regardless of N source. Importantly, 1 mM represented a N-sufficient condition, since raising NO$_3^-$ supply to 2.5 mM did not further increase plant biomass (Fig. 1). However, when NH$_4^+$ was raised to 2.5 mM plants displayed moderate symptoms of ammonium toxicity in terms of slower growth (Fig. 1).

A high NH$_4^+$ concentration in the medium is known to affect the homeostasis of essential cations in the cell. For instance, NH$_4^+$ affects K$^+$ transport directly by competitive inhibition and indirectly via effects on membrane potential (Coskun et al. 2017). Moreover, NH$_4^+$ may also stimulate K$^+$ efflux (Coskun et al. 2017). Among others, a decrease in K$^+$ levels has been observed in grasses such as ryegrass (Belastegui-Macadam et al. 2007), rice (Balkos et al. 2010) or sorghum (Miranda et al. 2017). Indeed, when the available concentration of K$^+$ in the nutrient solution is limiting, the provision of supplementary K$^+$ improves ammonium tolerance (Balkos et al. 2010; Li et al. 2012). In contrast, this is not the case when the concentration of K$^+$ is already sufficient (Balkos et al. 2010). In our work, despite the fact that we report a significant decrease in Ca, K and Mg content in the root of plants grown with 2.5 mM NH$_4^+$ [see Supporting Information—Table S4], the internal concentrations of these elements remained within the limits of an adequate nutrient supply (Marschner 2012). Therefore, under our growth conditions, ion imbalance does not seem to be the primary cause for the growth inhibition observed in B. distachyon fed with 2.5 mM NH$_4^+$. However, further experiments are needed to completely discard the potential role of cationic imbalance in the response of B. distachyon to ammonium toxicity.

The main metabolic symptom of ammonium toxicity, and the probable cause of subsequent disorders, is the disruption of cytosolic NH$_4^+$ homeostasis. Thus, one of the obvious cell strategies for keeping NH$_4^+$ cytosolic levels under control is to intensify its assimilation into organic molecules. It has already been reported that a prevalent response of many species, including grasses, is
an accumulation of free amino acids primarily in the roots, e.g., in wheat and sorghum (Setién et al. 2013; Miranda et al. 2016). Alternatively, other species such as rapeseed (Coleto et al. 2017) tend to accumulate amino acids in shoot tissues. The amino acids in which NH₄⁺ is preferentially stored also vary between species. As in other monocots such as wheat (Setién et al. 2013), NH₄⁺ is scavenged in *B. distachyon* root systems in the form of amides, mainly Asn followed by Gln (Fig. 3).

Glutamine synthetase/glutamate synthase is the main NH₄⁺ assimilation pathway and its importance during ammonium...
stress has been highlighted by the fact that mutants such as rice gln1;1 or Arabidopsis gln1;2 mutants show hypersensitivity to ammonium nutrition (Kusano et al. 2011; Guan et al. 2016). In B. distachyon Bd21, GS is encoded by four genes [see Supporting Information—Table S1, Fig. S4]. Various studies have revealed that GS is regulated at multiple levels: transcriptional, translational and post-translational. Moreover, GS members are differentially regulated (i) in space, according to cell type and function of the organ; (ii) in time, according to circadian rhythm and phenology; and (iii) in response to growth conditions...
(Swarbreck et al. 2011). Accordingly and in contrast to the decrease of GS activity observed in roots of plants fed with ammonium (Fig. 3), the expression of BdGLN1;1 and BdGLN1;2 did not vary and only BdGLN1;3 expression was enhanced by NH₄⁺ (Fig. 4A) thereby underlining the probable post-translational regulation of GS. A further and interesting finding is that in roots of barley, HvGS1.3, homologue of BdGLN1;3 was also induced by NH₄⁺ nutrition (Goodall et al. 2013), indicating that the function and regulation of these isogenes can be similar for both monocot species. Thus, it will be of interest to explore whether the role of BdGLN1;3 is significant under ammonium stress. The decrease in root GS activity observed in plants fed with ammonium (Fig. 3) could be related to a feedback regulation by the accumulation of amino acids (Watanabe et al. 1997; Oliveira and Coruzzi 1999) as represented by the high Asn/Asp and Gln/Glu ratios (Fig. 3; see Supporting Information—Fig. S3, Table S3).

Asparagine synthetase (AS) catalyses the ATP-dependent synthesis of Asn and Gln from Gln and Asp. Asn has a high ratio N:C and plays a key role in nitrogen transport and storage (Lea et al. 2007). BdASN1 and BdASN3 were induced by ammonium treatment (Fig. 4C) and thus, probably control Asn synthesis in B. distachyon fed with ammonium. Indeed, in plants fed with 2.5 mM NH₄⁺, Asn accounted for ca. 51 and 70 % of total leaf and root amino acid content, respectively (see Supporting Information—Tables S2 and S3). The higher expression of BdASN1 and its specific up-regulation by ammonium nutrition in roots suggest a prominent role of this gene when ammonium is the only source of N (Fig. 4C). In support of this, OsASN1, a homologue of BdASN1 (see Supporting Information—Fig. S6), was previously shown responsible for Asn accumulation in rice grown under ammonium nutrition (Ohashi et al. 2015).

To sustain amino acid synthesis in the root an adequate supply of carbon is essential, necessitating adjustments in carbon metabolism elsewhere. Specifically, TCA cycle adjustment has been shown to be crucial, as well as its associated anaplerotic routes (Setién et al. 2014; Sarasketa et al. 2016). The significant effect of ammonium nutrition on PEPC activity (Fig. 6) was presumably essential to guarantee the flux of carbon towards OAA that can yield Asp or follow the Krebs cycle. Moreover, ME activity would also collaborate, through pyruvate entrance in the cycle, in the provision of citrate + isocitrate to generate 2-OG in co-ordination with ICDH (Fig. 6). Indeed, low malate and PEP contents indicate a higher consumption under ammonium as a result of citrate entering the TCA cycle, in the provision of citrate + isocitrate to generate 2-OG. Furthermore, ME activity would also collaborate, through NADH regeneration, in the delivery of electrons for the reduction of NAD⁺/NADP⁺. Overall, these observations indicate that B. distachyon to ammonium nutrition are in line with previous studies with cereals crops. Our work underlines the potential of B. distachyon as a useful tool for analysing the molecular basis of ammonium tolerance in monocots. Such work is of paramount importance in view of the desirability of increasing the use of ammonium-based fertilizers to lessen environmental pollution associated with nitrate-based nutrition.

**Data**

The raw data that support the findings of this study are available from the corresponding author, upon reasonable request.

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**Contributions by the Authors**

D.M. and M.B.G. conceived the study and supervised the project; M.P. and D.M. performed experiments. M.P., M.B.G and D.M. analysed data. M.P. and D.M. wrote the paper and, all authors edited and approved the final manuscript.

**Conflict of Interest**

None declared.

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Supporting Information

The following additional information is available in the online version of this article—

Figure S1. Nitrate contents in leaves and roots of Brachypodium distachyon grown with nitrate or ammonium as N source.

Figure S2. NADP(H)-GDH (A) and NADP-dependent ME (B) enzyme activities in leaves and roots of Brachypodium distachyon grown with nitrate or ammonium as N source.

Figure S3. Gin/Glu (A) and Asn/Asp (B) ratios in leaves and roots of Brachypodium distachyon grown with nitrate or ammonium as N source.

Figure S4. Phylogenetic tree of glutamine synthetase (GS) genes.

Figure S5. Phylogenetic tree of glutamate dehydrogenase (GDH) genes.

Figure S6. Phylogenetic tree of asparagine synthetase (ASN) genes.

Figure S7. C/N ratio (A) and carbon content (B) of leaves and roots of Brachypodium distachyon grown with nitrate or ammonium as N source.

Table S1. Brachypodium distachyon Bd21 genes encoding for GLN, AS and GDH enzymes.

Table S2. Individual amino acid contents in leaves of Brachypodium distachyon grown with nitrate or ammonium as N source.

Table S3. Individual amino acid contents in roots of Brachypodium distachyon grown with nitrate or ammonium as N source.

Table S4. K, Ca, Mg and Na contents of leaves and roots of Brachypodium distachyon grown with nitrate or ammonium as N source.

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