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Ammonium tolerance and the regulation of two cytosolic glutamine synthetases in the roots of sorghum

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Abstract. Tolerance to ammonium nutrition in plants can be related to their ability to detoxify ammonium via nitrogen assimilation in roots. Here, we report that sorghum–sudangrass (*Sorghum bicolor* L. × *S. bicolor* var. sudanense) hybrids exhibited enhanced biomass production under high levels of inorganic nitrogen supply as well as increased capacity for nitrogen assimilation in roots. Glutamine synthetase (GS, EC 6.3.1.2) activity and protein accumulated in roots at increasing concentrations of either nitrate or ammonium, with particularly high levels of GS in ammonium-treated plants. Ammonium but not nitrate differentially regulated two distinct cytosolic GS (GS1) isoforms composed by polypeptides of similar size but different charge. The comparative analysis of GS gene sequences and the deduced GS1 polypeptides suggested that the two GS1 isoforms were the expression products of *SbGln1.2* and *SbGln1.3* genes. *SbGln1.3* expression was shown to be upregulated by high levels of inorganic nitrogen supply, with a maximal abundance of *SbGln1.3* transcripts in ammonium-grown plants. *SbGln1.2* expression was uniform along the root axis meanwhile protein and transcript levels for *SbGln1.3* were particularly abundant in the upper part of the axis where lateral roots are prominent. Kinetic analysis revealed that the two GS1 isoenzymes have relatively low-affinity for ammonium ions. The spatial distribution of low-affinity GS1 isoenzymes would provide a sustained glutamine biosynthesis at high levels of ammonium supply and may represent at the same time an efficient system of ammonium detoxification. Such a mechanism may prevent transport of ammonium to leaves alleviating symptoms of toxicity and therefore contributing to sorghum ammonium tolerance.

Additional keywords: biomass production, nitrate, nitrogen assimilation.

Introduction

Nitrogen is quantitatively the most essential nutrient for plants and a major limiting factor in plant growth and crop productivity and yield (Hirel *et al*. 2007; Tabuchi *et al*. 2007). Along the evolutionary history of higher plants, several strictly regulated metabolic processes have evolved to guarantee nitrogen acquisition and assimilation. Most plants are able to take up inorganic nitrogen available in the soils either in the form of nitrate or ammonium ions. Following absorption by the root, nitrate may be reduced in the roots or the shoots to generate ammonium by the sequential action of nitrate and nitrite reductases. Ammonium is the form of inorganic nitrogen ultimately assimilated through glutamine synthetase (GS, EC 6.3.1.2), a key enzyme in N metabolism and plant productivity (Lea and Ireland 1999). GS catalyses the incorporation of ammonium into the amide group of glutamine at expenses of ATP hydrolysis. Recently, it has been reported that plant GS holoenzyme has a decameric structure composed of two face-to-face pentameric rings of identical subunits (Unno *et al*. 2006). In angiosperms, there are two major isoenzymes of GS located in different subcellular compartments and displaying non-overlapping roles: (1) GS1, mainly found in the cytosol of cells associated to the vascular elements and (2) GS2, located in the stroma of chloroplasts. GS2 is responsible for the assimilation of ammonium derived from nitrate reduction and photorespiration (Ireland and Lea 1999) whereas GS1 is the predominant enzyme in roots and non-photosynthetic tissues and much less abundant in green tissues (Ireland and Lea 1999). GS1 seems to be involved in the primary assimilation of ammonium from the soil and the recycling of ammonium released through metabolic processes such as protein remobilisation and phenylpropanoid biosynthesis (Cantón *et al*. 2005; Tabuchi *et al*. 2007). In conifers, the reaction catalysed by cytosolic glutamine synthetase (GS1) is a key step controlling the plant capacity to assimilate and recycle nitrogen (Cánovas *et al*. 1998, 2007). Cytosolic GS is encoded by a small gene family whose members are expressed differentially during development and in response to environmental stimuli, gives rise to a variable number of GS1 isoforms (Forde and

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Cullimore 1989; Cren and Hirel 1999) while a single nuclear gene encode chloroplastic GS in most plants. Several reports in the last decade supported a role of GS1 in plant development, growth and biomass production (Fuentes *et al*. 2001; Oliveira *et al*. 2002; Jing *et al*. 2004). Genetic and molecular approaches have shown that GS may be a key component of plant N use-efficiency and yield in higher plants (Hirel *et al*. 2001; Yamaya *et al*. 2002; Tabuchi *et al*. 2005; Martin *et al*. 2006).

Sorghum (*Sorghum bicolor* L.) is an important staple food crop in developing countries and was ranked the seventh most important crop worldwide in terms of harvested area. It is the dietary staple for more than 500 million people in many countries and more than 50% of the global harvest takes place in Africa. Sorghum is also a well established model for molecular and physiological studies in photosynthesis and C metabolism (Bakrim *et al*. 1998; Nhiri *et al*. 1998). However, knowledge on N assimilation and metabolism is much more limited. For example, two isoforms of glutamine synthetase (GS1 and GS2) were characterised in sorghum leaves by Hirel and Gadal (1982) more than 25 years ago but the regulation of this key enzyme has not been further reassessed. In this work, we studied the regulation of GS in the roots of plants exposed to increased concentrations of nitrate and ammonium. We have used as experimental model sorghum–sudangrass hybrids (*S. bicolor* × *S. bicolor* var. sudanense), which are extensively used as a crop for their favourable characteristics such as ability to assimilate and stock excess of nitrogen, quick growth, nutritional quality and tolerance to heat and drought. Intensive growth of sorghum–sudangrass hybrids in short periods of water shortage (summer) requires the supply of high levels of inorganic nitrogen that is provided usually in the form of nitrate.

The application of excessive levels of nitrate enhances biomass production but leads to its accumulation in leaves where it can be toxic for animal and human health. Ammonium nutrition has been suggested as an alternative to nitrate nutrition that is limited by its toxicity to plants with reported symptoms such as leaf chlorosis, suppression of growth and a lowering of root : shoot ratios (Britto and Kronzucker 2002). The current model of ammonium toxicity explains that these symptoms are the consequences of ionic imbalance, metabolic and hormonal alterations (Britto and Kronzucker 2002). However, sorghum–sudangrass hybrids exhibited enhanced plant growth when plants were exposed to moderate and high levels of ammonium. It has been proposed that tolerance to ammonium can be related to the plant's capacity to maintain high levels of inorganic nitrogen assimilation in the roots (Cruz *et al*. 2006). Assimilation of ammonium in the roots by GS would prevent its accumulation and transport to the shoot where the toxic effects are much more important. The potential role of root GS1 enzymes in the regulation of sorghum ammonium tolerance is discussed.

Materials and methods

Plant material and growth

Sorghum–sudangrass hybrid seeds (*Sorghum bicolor* L. × *S. bicolor* var. sudanense) were sterilised with 5% of NaOCl for 15 min and washed thoroughly with sterile water and germinated in a pot with vermiculite as supporting substrate. Seedlings were then grown in a controlled growth chamber at 25°C, with a 14-h photoperiod. Illumination was provided by fluorescent lamps (Sylvania F-48T12/CW/WHO; Koxka, Pamplona, Spain) at a flux density of 150 µmol m^{-2} s^{-1}. Twice a week, plants received 100 mL per pot of a nutrient solution containing 0.5 mM KNO_3, 0.375 mM KH_2PO_4, 0.125 mM K_2HPO_4, 0.375 mM $MgSO_4$, 0.1 mM NaCl, 1.25 mM $CaSO_4$, 10 mg L^{-1} Fe-EDTA and micronutrients (Arnon 1938), pH 6.4 (±0.1). For nitrogen treatments, the nutrient solution was the same except that different concentrations of KNO_3 or $(NH_4)_2SO_4$ (5, 20, 50 mM) were used. Plants were harvested 20 days after the start of nutritional treatments and fresh weight and length were immediately measured. Sample aliquots were taken apart and dried in an oven at 80°C for 48 h for dry weight determination. Roots were quickly and gently washed with deionised water to remove residual vermiculite before protein and RNA extractions.

Protein extraction and determination

Root material was immediately frozen in liquid nitrogen after harvesting and stored at −80°C until use. Frozen tissues were ground in liquid nitrogen in a mortar with pestle and the resulting powder was transferred into a tube containing extraction buffer (50 mM Tris–HCl pH 8.0, 5 mM $MgSO_4$, 12 mM glutamate, 2 mM EDTA, 10% (v/v) glycerol, 0.1% (v/v) 2-mercaptoethanol). Extracts were filtered through miracloth into test tubes. The resulting homogenates were centrifuged twice at 15 000*g* for 30 min at 4°C and supernatant was removed to a new tube. Protein contents in the different samples were determined by the Bradford's method using bovine serum albumin as a standard (Bradford 1976).

GS activity measurements

Glutamine synthetase activity was routinely measured using the transferase assay (Cánovas *et al*. 1991). The assay mixture consisted of 90 mM imidazole-HCl (pH 7.0), 120 mM L-glutamine, 3 mM $MnCl_2$, 0.4 mM ADP, 20 mM sodium arsenate, 60 mM NH_2OH and the enzyme solution in a final volume of 2.25 mL. L-glutamine was omitted in the blank test. The reaction was started by adding NH_2OH (freshly prepared, and neutralised to pH 7.0 with NaOH) and incubated at 37°C for 15 min. The reaction was stopped by adding 0.75 mL of a mixture (1 : 1 : 1) of 10% $FeCl_3.6H_2O$ (in 0.2 N HCl), 24% trichloroacetic acid and 5% HCl. The appearance of γ-glutamyl hydroxamate was measured at 540 nm.

In kinetic experiments, GS activity was determined using the spectrophometric assay that couples GS activity to the oxidation of NADH by pyruvate kinase and lactate dehydrogenase. Reaction mixtures included 90 mM Mops (pH 7.2), 125 mM sodium glutamate, 6.25 mM ATP, 22 mM $MgSO_4$, 1 mM phosphoenolpyruvate, 80 mM KCl, 0.5 mM NADH, pyruvate kinase (30 µg mL^{-1}), lactate dehydrogenase (25 µg mL^{-1}) (Roche, Barcelona, Spain) and the appropriate concentrations of NH_4Cl.

SDS–PAGE and western blot analysis

Crude enzymatic extracts were separated on 12.5% SDS–polyacrylamide gels, the resolved polypeptides were electrotransferred onto nitrocellulose membranes and the presence of GS polypeptides immunorevealed with specific antibodies. Immunolabelling was carried out essentially as described by Cánovas *et al*. (1991) using the antiserum raised against recombinant pine GS (Cantón *et al*. 1996). Subsequent detection of immunocomplexes was carried out by a peroxidase assay.

Two-dimensional gel electrophoresis

Two-dimensional gel electrophoresis was carried out as described previously (Avila *et al*. 1998). The isoelectrofocusing (IEF) slab gels were $80 \times 70 \times 1$ mm of a mixture consisting of 5% acrylamide, 8.3 M urea, 2.5% carrier ampholytes (Bio-Rad España, Madrid, Spain; pH 4–6). The IEF was performed at 200 V for 2.5 h. The second dimension electrophoresis was also performed in $80 \times 70 \times 1.5$ mm slab gels of 12.5% (w/v) polyacrylamide gels containing SDS as described above. The resolved proteins were then transferred to nitrocellulose membranes and immunodetection was performed essentially as described for 1D western blots.

Separation of GS isoforms by ion-exchange chromatography

The desalted protein extract from roots was loaded on a DEAE-Sephacel column (20 cm × 2 cm; Sigma-Aldrich, Madrid, Spain) pre-equilibrated with a buffer containing 50 mM Tris–HCl pH 8.0, 5 mM $MgSO_4$, 12 mM glutamate, 2 mM EDTA and 0.1% (v/v) 2-mercaptoethanol. A linear gradient of 0–300 mM KCl was used to elute the proteins at a flow rate of 50 mL h^{-1}. The GS active fractions were pooled and concentrated by inverse dialysis overnight against solid sucrose and stored at 4°C. GS activity in the different fractions was determined by the transferase assay as described by Gálvez *et al*. (1990).

RNA extraction, cDNA synthesis and real time-PCR

Total RNA was extracted according Zhihua Liao *et al*. (2004) with minor modifications. First-strand cDNA was synthesised by RT polymerase (Roche) using oligo p $(dT)_{15}$ as primer, according to the manufacturer's instructions. One microlitre of the cDNA first-strand reaction was used in a standard 50 µL PCR reaction Go Taq Flexi (Promega Biotech Ibérica, Madrid, Spain; 5X Green buffer, 2.5 mM dNTPs, 25 mM $MgCl_2$, 1.25 U Taq DNA polymerase) with 2 µM of the specific oligonucleotides. The oligonucleotides used were: *Sbgln1.3* forward (5′-CTGCCG GTGACGAAATA-3′) and *Sbgln1.3* reverse (5′-GCCATTATT CAGACTTTC-3′), *Sbgln1.2* forward (5′-GAAGCTTTCCTC CATCC-3′) and *Sbgln1.2* reverse (5′-GAGGCCATCCAATC GGC-3′) or *Sbgln1.1* forward (5′-ACGAGACCGCCGAC ATC-3′) and *Sbgln1.1* reverse (5′-GTGTTCTTGGCTCG TTA-3′). PCR conditions were 95°C for 2 min, followed by 25–30 cycles (95°C for 30s, 50°C for 30s, 72°C for 1 min) and finished with 72°C for 5 min final extension. PCR products were separated in 0.8% agarose gel and DNA was visualised with ethidium bromide. Quantification of PCR products was performed using the image J (ver. 1.36) program.

Results

High levels of inorganic nitrogen lead to enhanced biomass production of sorghum–sudangrass hybrids

Sorghum–sudangrass hybrids were grown for 20 days under low and high levels of inorganic nitrogen supply (see Fig. S1 available as an Accessory Publication to this paper). Enhanced biomass improvements (assessed as increased plant dry/fresh weight and height) were observed at high levels of nitrate (20 and 50 mM) when compared with control plants growing at 0.5 mM nitrate (Fig. 1). However, similar biomass production was found at moderate levels of nitrate (5 mM). The utilisation of ammonium as sole N source in the nutrient solution also resulted in enhanced values of dry/fresh weight and height when compared with those observed in control plants (Fig. 1). Optimal biomass accumulation was observed at 5 and 20 mM of ammonium supply. Plants growing under extremely high levels of ammonium (50 mM) exhibited similar biomass production than control plants (Fig. 1).

High levels of inorganic nitrogen induce the accumulation of GS in sorghum roots

We were interested to know whether the enhanced accumulation of biomass observed at high levels of inorganic nitrogen

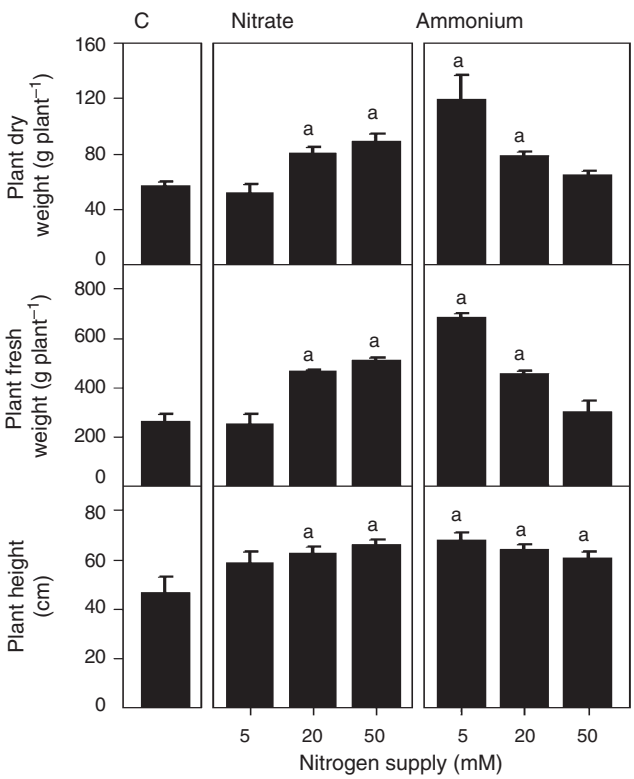

Fig. 1. Biomass accumulation of sorghum–sudangrass hybrids growing at increased levels of inorganic nitrogen supply. Plants were grown for 20 days under low nitrogen, 0.5 mM nitrate (C) or increased levels of nitrate and ammonium supply. Total dry/fresh weight and length values are the mean of at least 30 plants per treatment. Means were compared by Student's *t*-test. Significant differences at $P < 0.05$ between low nitrogen and treatments with increased levels of nitrate or ammonium are indicated by (a).

availability was supported by an increased capacity for nitrogen assimilation in the roots of these plants. In order to test this hypothesis, GS activity was determined in the roots of control plants growing at 0.5 mM nitrate and in those exposed to high concentrations of either nitrate or ammonium (5, 20, 50 mM). As shown in Fig. 2 (upper panel), total GS activity increased in response to the increased availability of inorganic nitrogen, and either nitrate or ammonium. The observed increase in GS activity could be explained by activation of a pre-existing enzyme or by the accumulation of GS protein in response to nitrogen availability. To figure out which of these two possibilities is operating in the effect mediated by nitrate/ammonium, western blot analyses were performed in the same extracts in which GS activity was previously measured (Fig. 2, lower panel). A GS polypeptide accumulated in the roots of sorghum at increasing concentrations of either nitrate or ammonium, with particularly higher levels of GS protein in ammonium-treated plants. The size of the GS polypeptide (40 kDa) was the same in control and nitrate- or ammonium-grown plants and it was in the range of the molecular mass described for cytosolic GS (GS1) in plants (Cren and Hirel 1999; Bernard and Habash 2009). A minorly represented and faster migrating polypeptide (38 kDa) was also detected in protein extracts from ammonium-grown plants. This weak cross-reacting band may represent a GS degradation product of the intact and highly abundant GS polypeptide. Taken together, these results suggest that high levels of inorganic nitrogen induce the accumulation of GS1 protein and activity in sorghum roots.

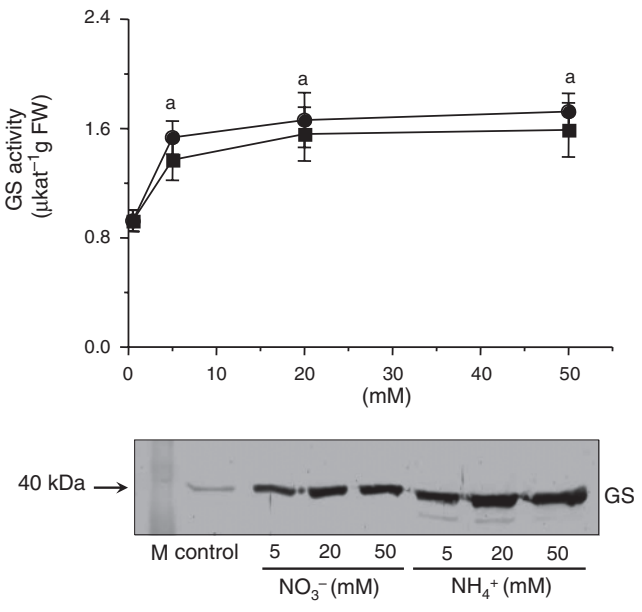

Fig. 2. GS activity and GS protein abundance in the roots of sorghum–sudangrass hybrids grown under increased levels of nitrate and ammonium supply. Nitrate (■); ammonium (●). GS activity values are the mean ± s.d. of four independent determinations. Means were compared by Student's *t*-test. Significant differences at $P < 0.05$ between low nitrogen and treatments with increased levels of nitrate or ammonium are indicated by (a). M, markers of molecular size. Control sample corresponds to plants grown under low nitrogen (nitrate 0.5 mM). The size of the GS polypeptide (40 kDa) is indicated on the left. The same amount of protein was loaded per lane in the gels.

Ammonium but not nitrate regulates the accumulation of two distinct GS1 isoforms in sorghum roots

GS1 is encoded by a family of several genes ranging 2–5 members depending of the plant species examined (Cren and Hirel 1999; Bernard and Habash 2009). Consequently, we were interested to know whether a single GS1 or more that one GS1 isoform accumulates in sorghum roots in response to high nitrogen availability. To specifically investigate this point, soluble protein extracts from control, nitrate and ammonium-treated plants were separated by 2D gel electrophoresis (2D-GE) and the presence of GS polypeptides in the samples immunorevealed by western blot analysis (Fig. 3). Two different spots (a and b) of the same size but different charge and relative abundance were identified in plants grown at low nitrate (Fig. 3, control). A similar proportion of the two GS1 polypeptides was observed in plants exposed to high levels of nitrate (Fig. 3, nitrate). However, and in contrast to the results obtained in nitrate-grown plants, the proportion of the acidic GS1 polypeptide (a) dramatically increased in ammonium-grown plants (Fig. 3, ammonium). Note that in agreement with the results shown in Fig. 2, the relative abundance of GS immunoreactive spots was greater in ammonium-treated plants (Fig. 3, ammonium).

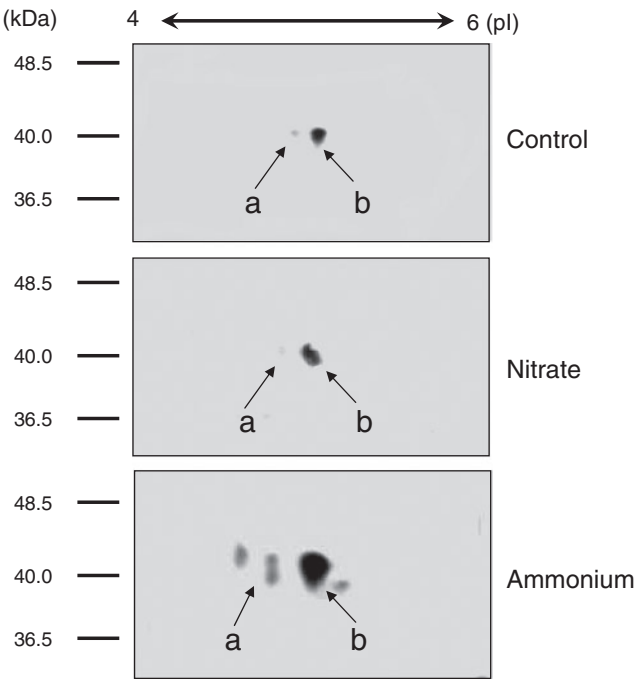

Fig. 3. Separation and identification of GS1 polypeptides in the roots of sorghum–sudangrass hybrids by 2D-GE and western blot analysis. Samples were from plants grown under 0.5 mM nitrate (control), nitrate (50 mM) or ammonium (50 mM). Major acidic (a) and basic (b) spots observed in the different experimental conditions are marked by arrows. The spot variants possibly correspond to allelic differences since several plants were sampled for each experiment. Post-translational modifications could also be considered (Finnemann and Schjoerring 2000).

To examine whether ammonium supply was able to specifically induce the accumulation of a barely detectable GS1 isoform in sorghum roots, GS activity was fractionated through ion-exchange column chromatography. Crude extracts containing total GS activity from roots of nitrate- and ammonium-treated plants were loaded on the top of DEAE-Sephacel columns. After extensive washing of the columns, the enzyme activity was eluted by the application of a linear gradient of KCl (Fig. 4). When protein extracts from nitrate-treated plants were fractionated, a single peak of GS was detected, which eluted from the column at 0.15 M of KCl (Fig. 4, nitrate). In contrast, two peaks of GS activity (peak I and peak II) were resolved when proteins extracts from ammonium treated plants were analysed (Fig. 4, ammonium). Peak I eluted from the column at a similar KCl concentration (0.16 M) to that recorded for the unique GS isoform detectable in control plants; however, peak II eluted from the column at a higher ionic strength (0.2 M KCl).

To explore the composition in GS polypeptides of the two isoenzymes separated by ion-exchange chromatography, the fractions of the two peaks were pooled, concentrated and analysed by 2D-GE and western blotting (Fig. S2). In a set of experiments the GS1 isoforms were analysed separately loading similar amounts of protein in the gels (Fig. S2, upper panels, peaks I and II). Peak I was shown to contain almost exclusively the basic polypeptide identified in Fig. 3 (spot b) whereas peak II was composed mainly by the acidic polypeptide (spot a). As sorghum–sudangrass hybrids should contain allelic forms of GS1 polypeptides that could mask the interpretation of data, we performed a second set of experiments in which equal amounts of GS activity from peaks I and II were mixed and loaded in the gels to facilitate the identification of the relevant GS1 polypeptides (Fig. S2, lower panel, peak I + peak II). Two major polypeptides displaying equivalent abundances were clearly separated: the acidic (a) had a calculated IP of 5.04 whereas the experimentally determined IP value for the basic one (b) was 5.26. Minor spots observed could correspond to GS allelic variants and/or post-translational modifications of the GS1 polypeptides (Finnemann and Schjoerring 2000).

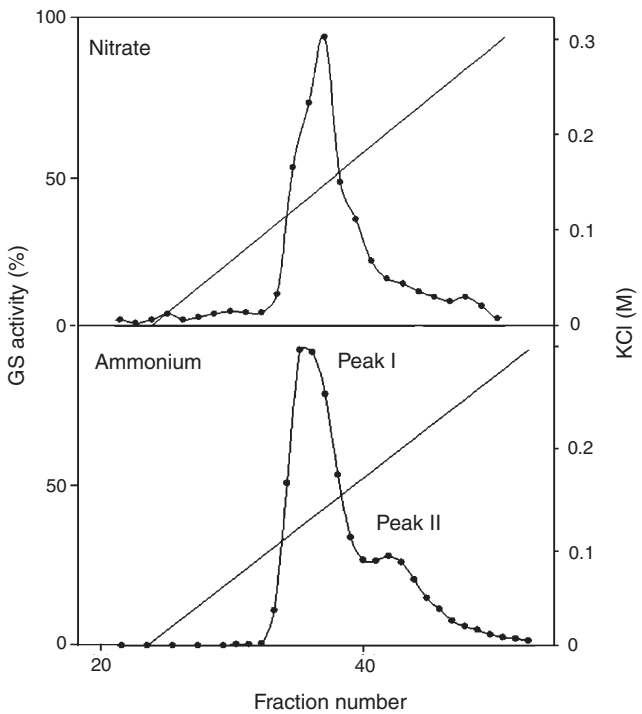

Fig. 4. Separation of GS1 isoforms by ion-exchange chromatography root extracts of sorghum–sudangrass hybrids grown under nitrate or ammonium supply. A single peak eluted from the column at 0.15 M KCl in nitrate-grown plants (upper panel). Two peaks: peak I (0.16 M KCl) and peak II (0.2 M KCl) eluted from the column in ammonium-treated plants. Maximum GS values were 91 nkatal ml^{-1} and 334 nkatal ml^{-1} in upper and lower panels, respectively.

Nitrogen regulated expression of GS1 genes in sorghum roots

Since distinct genes encode GS1 polypeptides in many plant species, it was interesting to examine which members of the sorghum GS gene family are expressed in roots and regulated by ammonium. To reach this goal, we performed a bioinformatic analysis of the available GS sequences in the databases. In the last few years, sequencing projects have been initiated for several crop species of cereal grasses (Devos 2005) (http://www.phytozome.net/, accessed 7 June 2008). Furthermore, the 730-megabase of the *S. bicolor* genome has been recently analysed (Paterson *et al*. 2009). The availability of these genomic resources enables us to compare the GS sequences present in the EST databases with those identified in the *Arabidopsis thaliana* (L.) genome and the GS family of the C4 plant sugarcane (*Saccharum officinarum* L.) (Nogueira *et al*. 2005). A total number of 23 annotated GS ESTs were identified with a high similarity to individual *Arabidopsis* GS genes and covering in some extent the primary structure of individual sorghum isoforms (Table 1). According to our analysis, a single gene for GS2 (*SbGln2*) and three genes for GS1 (*SbGln1.1*, *SbGln1.2*, *SbGln1.3*) were shown to be expressed in *S. bicolor*. These data are supported by the occurrence of two GS isoforms (GS1 and GS2) in the leaves and GS1 in roots (Fig. S3) and are also in close agreement with the pioneering report by Hirel and Gadal (1982) describing GS1 and GS2 isoforms in the leaves of *Sorghum vulgare* L. It is interesting to note that three functionally distinct subfamilies of cytosolic GS have been reported in *Poaceae* species (Bernard and Habash 2009). The sequence information derived from the individual genes described in Table 1 allowed us the design of specific oligonucleotides to monitor the expression levels of GS1 genes by RT-PCR. Thus, the relative abundance of transcripts for the three GS1 genes (*SbGln1.1*, *SbGln1.2* and *SbGln1.3*) was investigated in sorghum roots (Fig. 5). *SbGln1.2* was the major gene expressed at low nitrate (Fig. 5, control). However, the exposure to high levels of either nitrate or ammonium markedly induced the accumulation of *SbGln1.3* transcripts (Fig. 5, nitrate and ammonium). Interestingly, *SbGln1.3* gene expression was shown to be particularly upregulated by ammonium. Fig. 5 also shows that *SbGln1.1* transcripts were poorly represented in sorghum roots, at least in our experimental conditions.

Specific oligonucleotides were also used for the amplification of a full-length cDNA for *SbGln1.3* containing the complete

Table 1. *Sorghum bicolor* sequences with similarity to glutamine synthetase genes in plants

Gene	Accession number	Isoenzyme	Location	Major expression
SbGln2	TC111545 TC124212 TC131002 BE365021	GS2	Plastid	Leaves
SbGln1.1	TC113201 TC11888 TC101935 TC103694	GS1	Cytosol	Leaves, embryos, stress, callus culture
SbGln1.2	TC112461 TC113183 TC119926 TC119535 TC122596 TC113183 CD462839 CD205511 AW671987	GS1	Cytosol	Roots, pathogen induced, N deficiency
SbGln1.3	TC111441 TC112590 TC102551 TC132978 CD429292	GS1	Cytosol	Roots, seedlings, wounded leaves

ORF encoding the GS polypeptide. The complete sequence was deposited in the EMBL database under the accession number FM196538. The deduced GS1 polypeptide had a pI value of 5.0, quite similar to the experimental pI value of the acidic GS1 isoform (5.04). The pI of the basic GS isoform (5.26) was also quite similar to the deduced value of the GS1 polypeptide encoded by the *SbGln1.2* cDNA (5.30).

The two GS1 isoforms expressed in sorghum roots exhibit low-affinity for ammonium ions

The kinetic analyses of GS1 isoforms in plants revealed the existence of two different groups with regard to their affinities for the ammonium ions: low-affinity (with K_m values ranging from 200 to 2500 μM) and high-affinity isoenzymes (with K_m values from 10 to 80 μM) (Sakakibara *et al*. 1996; Ishiyama *et al*. 2004*a*; Tabuchi *et al*. 2007). In order to determine the enzymatic properties of the GS1 isoforms expressed in sorghum roots, partially purified preparations of the two enzymes were used to determine the saturation curves for ammonium and K_m values were calculated to be 170 and 270 μM for the acidic and basic GS1 isoforms, respectively (Fig. S4).

The two GS1 isoforms are differentially expressed along sorghum roots

We were interested to know whether or not the two GS1 isoforms were uniformly distributed in sorghum roots in response to ammonium treatment. Four different sections (1 to 4) from the lower to the upper part of the roots were analysed by 1D- and 2D-GE, and western blotting (Fig. 6). The relative abundance of GS1 protein increased from the tip (1) to the root–stem junction (4) where the number of lateral roots was particularly prominent

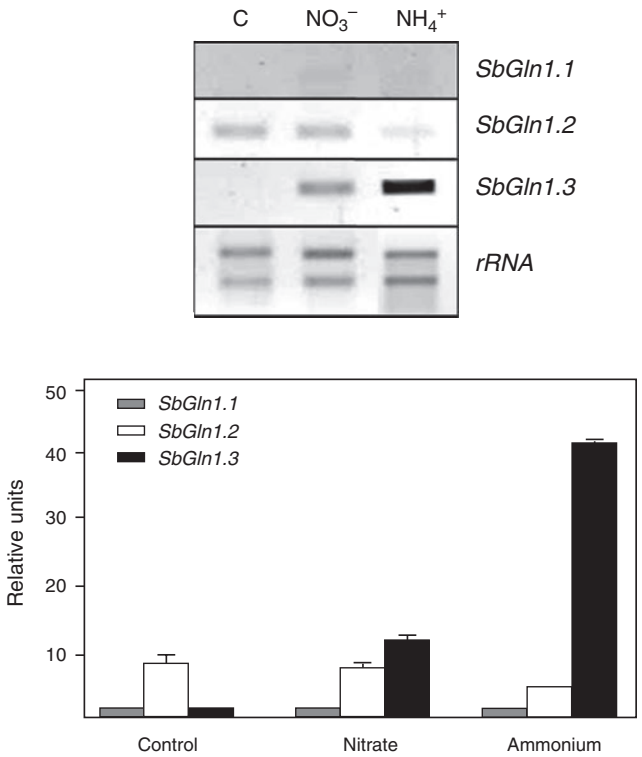

Fig. 5. Expression analysis of *SbGln1.1*, *SbGln1.2* and *SbGln1.3* genes in the roots of sorghum–sudangrass hybrids grown under low nitrogen (control) or increased levels of nitrate and ammonium supply (50 mM). Total RNA was isolated from roots and reverse transcription was performed in the presence of oligodT oligonucleotide. The first-strand cDNA was amplified by PCR using specific primers for *SbGln1.1*, *SbGln1.2* and *SbGln1*.3 transcripts as described in 'Materials and methods'. Values are the mean ± s.d. of at least three independent determinations.

(Fig. 6*a*). The separation of GS1 isoforms by 2D-GE showed that the accumulation of GS protein along the root was mainly attributed, if not exclusively, to the accumulation of the acidic GS1 polypeptide (Fig. 6*b*).

The relative abundance of transcripts for the two GS1 genes expressed in roots (*SbGln1.2* and *SbGln1.3*) was also determined in the same sections in which GS1 polypeptides were previously separated (Fig. S5). The steady-state levels of *SbGln1.2* mRNA were quite similar in sections 1 and 2 with slightly lower abundance of transcripts in sections 3 and 4. In contrast, the relative abundance of *SbGln1.3* mRNA increased significantly from the tip (section 1) to the upper part of the root (section 3) where the lateral roots were more abundant (Fig. 6*b*). Similar levels of *SbGln1.2* and *SbGln1.3* transcripts were observed in section 4. The enhanced expression of *SbGln1.3* was well correlated with the increase of GS1 (a) polypeptide along the root except the upper part of the organ close to the stem.

Discussion

Sorghum–sudangrass hybrids are able to growth and yield enhanced levels of plant biomass under high levels of either nitrate or ammonium nutrition (Fig. 1). Furthermore, plants supplied with extremely high concentrations of ammonium

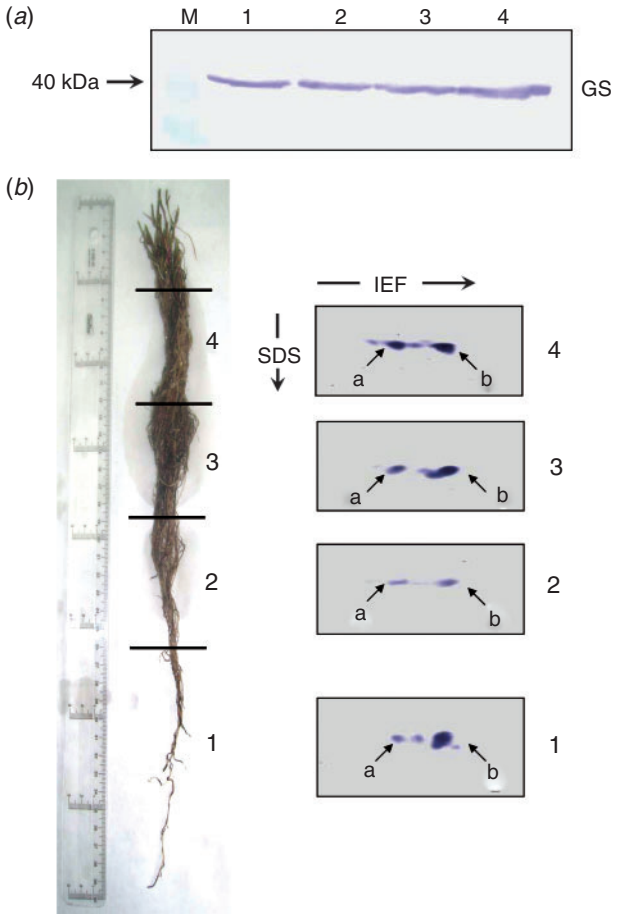

Fig. 6. Distribution of GS polypeptides in different sections of sorghum roots (1 to 4). (*a*) 1D analysis, (*b*) 2D analysis.

(50 mM) exhibited similar biomass production than those supplied with low and moderate levels of nitrate (0.5–5 mM) and did not show apparent signs of toxicity. Only a few plants are able to growth and accumulate biomass with ammonium as the sole nitrogen source without exhibiting severe toxicity symptoms such as chlorosis of leaves and suppression of growth (Britto and Kronzucker 2002). A critical factor in most plants showing ammonium tolerance is their ability to detoxify ammonium via the enzymatic action of GS activity in the roots (Givan 1979; Cruz *et al*. 2006). In this study, we report that the ability of sorghum–sudangrass hybrids to yield enhanced biomass at high levels of inorganic nitrogen nutrition is well correlated with the increased GS activity and the specific regulation of GS1 isoforms in roots.

We have found that high levels of either nitrate or ammonium trigger the accumulation of GS activity and GS1 protein in sorghum roots; however, the effect was more pronounced with ammonium (Fig. 2). These differences might be explained by an indirect effect of nitrate mediated after its reduction to ammonium in roots. In fact, we have detected increased levels of nitrate reductase activity in the roots of nitrate-fed sorghum plants (results not shown). It has been reported in maize (*Zea mays* L.) that the majority of ammonium absorbed from soil is assimilated in roots whereas nitrate is mainly assimilated in the leaves (Murphy and Lewis 1987). Nevertheless, increased nitrate assimilation in the roots has also been reported in ammonium tolerant plants when increased levels of nitrate were supplied (Cruz *et al*. 2006).

Our data demonstrate that increased ammonium nutrition induces the accumulation of two different GS1 polypeptides of the same size but differing in charge: acidic (a) and basic (b). However, increased nitrate nutrition apparently did not provoke the accumulation of the acidic (a) GS1 polypeptide (Fig. 3). Ammonium-specific induction of GS isoforms has been reported in the roots of several plant species including soybean (*Glycine max* L.) (Hirel *et al*. 1987), maize (Sakakibara *et al*. 1996), *Arabidopsis* (Ishiyama *et al*. 2004*a*) and rice (*Oryza sativa* L.) (Ishiyama *et al*. 2004*b*). In soybean roots, the expression of genes encoding cytosolic GS is induced by ammonium provided externally or by symbiotic nitrogen fixation (Hirel *et al*. 1987). The occurrence of two ammonium-regulated GS1 isoforms has been reported in the roots of rice (Ishiyama *et al*. 2004*b*), a crop plant usually cultivated in paddy fields that utilise ammonium as a major nitrogen source. These GS1 isoforms are encoded by separate genes, which showed differential responses to ammonium in specific cell types of rice roots (Ishiyama *et al*. 2004*b*). Similarly, two cytosolic GS isoforms were identified in maize roots but only one was responsible of the observed GS activity enhancement in response to an increase in the ammonium supply (Sakakibara *et al*. 1996). In contrast, four GS1 isoenzymes are expressed in the roots of *Arabidopsis* of which only one was significantly upregulated by ammonium (Ishiyama *et al*. 2004*a*).

In the present work, combined experiments of ion-exchange chromatography, 2D-GE, western blotting and expression analysis were used to determine that ammonium specifically induces the accumulation of a functional GS1 isoenzyme exclusively constituted by the acidic (a) GS1 polypeptide (Figs 4, 5; Fig. S2). Furthermore, our data indicate that the accumulation of this particular GS1 isoform is mainly located in the upper part of the root where a high number of lateral roots are present (Fig. 6).

The bioinformatic analyses of GS sequences in the *Sorghum bicolor* Database revealed that four individual GS genes are expressed in a variety of tissues and physiological conditions (Table 1). *SbGln2* encodes the GS2 isoenzyme whereas three different cDNAs encoding GS1 isoforms were identified (*SbGln1.1*, *SbGln1.2* and *SbGln1.3*). These findings are in close agreement to those reported for the GS gene family of sugarcane (Nogueira *et al*. 2005). However, the existence of additional GS genes in the sorghum genome with a low level of expression cannot be excluded. In fact, a higher number of GS genes are present in the sequenced genomes of *Arabidopsis* and poplar (*Populus trichocarpa* (Torr. & Gray)). Gene expression analyses indicated that *SbGln1.1* transcripts were barely abundant in sorghum roots and consequently, the two distinct GS1 isoforms characterised in the present work should be the expression products of *SbGln1.2* and *SbGln1.3* genes. This conclusion is further supported by the close similarity between the pI values of the acidic (a) and basic (b) GS1 isoforms and the corresponding GS1 polypeptides deduced from *SbGln1.3* and *SbGln1.2*, respectively. Our data suggest that *SbGln1.3* encodes

a functional GS1 isoenzyme that specifically accumulates in sorghum roots in response to ammonium supply, and mainly in the root zone where lateral roots are emerging (Fig. 6). Furthermore, our results also suggest that the ammonium- and spatial-dependent accumulation of this GS1 in roots appears to be, at least partially, controlled at transcriptional level. Nitrate appears to have an indirect effect on *SbGln1.3* expression after its reduction to ammonium. Thus, the lower levels of gene expression under these nutritional conditions further support a transcriptional control of GS accumulation mediated by ammonium. It remains to be determined whether or not the promoter region of *SbGln1.3* contains *cis*-regulatory elements involved in such transcriptional regulation. In this context, it is known that a strong constitutive positive element located in the distal part of the soybean *GS1* gene (*GS15*) is essential for ammonium regulation and subjected to modulation by other *cis*-regulatory sequences (Tercé-laforgue *et al*. 1999). The nature of the trans-acting factors specifically involved in the ammonium response of GS genes is presently unclear.

The two GS1 isoforms differentially expressed in the roots of sorghum had relatively low-affinity for ammonium suggesting they are able to assimilate high levels of ammonium regulating and maintaining a sustained biosynthesis of glutamine at high levels of ammonium supply. This amino acid can be either utilised for the biosynthesis of nitrogen compounds in the roots or exported via the vascular cylinder to the shoots. In *Arabidopsis*, low and high affinity isoforms are regulated in response to nitrogen availability (Ishiyama *et al*. 2004*a*). In contrast, low-affinity GS1 isoforms were absent in rice roots (Ishiyama *et al*. 2004*b*), a plant species adapted to ammonium nutrition. Taken together, these findings suggest that when high levels of ammonium ions are available, glutamine biosynthesis in sorghum roots is regulated by the spatial distribution of GS1 isoforms with a similar affinity for ammonium more than by the expression of individual enzymes exhibiting differential kinetics. Nevertheless, the expression of a high-affinity GS1 isoform in sorghum roots could not be excluded in other nutritional situations that have not been examined in the present work, for example in nitrogen limiting conditions.

Another point that should be considered is how ammonium transporters are arranged to coordinate ammonium influx into roots. Yuan *et al*. (2007) recently reported that the organisation of high-affinity ammonium uptake in *Arabidopsis* roots depends on the spatial arrangement of AMT1-type transporters. Aquaporins located in the root plasma membrane may also have a role in low-affinity ammonium transport and accumulation in root vacuoles (Loqué *et al*. 2005). Thus, the distribution of individual ammonium transporters/permeases in the cells of lateral roots could control the availability of high levels of ammonium and the subsequent accumulation of the *SbGln1.3* protein product in these plant tissues. The storage of ammonium in roots and subsequent assimilation by low-affinity GS1 isoenzymes may represent an efficient system of ammonium detoxification. Such a mechanism may prevent transport of ammonium to leaves alleviating symptoms of toxicity and therefore contributing to sorghum ammonium tolerance.

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