FEATURED ARTICLE

Soybean ureide transporters play a critical role in nodule development, function and nitrogen export

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SUMMARY

Legumes can access atmospheric nitrogen through a symbiotic relationship with nitrogen-fixing bacteroids that reside in root nodules. In soybean, the products of fixation are the ureides allantoin and allantoic acid, which are also the dominant long-distance transport forms of nitrogen from nodules to the shoot. Movement of nitrogen assimilates out of the nodules occurs via the nodule vasculature; however, the molecular mechanisms for ureide export and the importance of nitrogen transport processes for nodule physiology have not been resolved. Here, we demonstrate the function of two soybean proteins – GmUPS1-1 (XP_003516366) and GmUPS1-2 (XP_003518768) – in allantoin and allantoic acid transport out of the nodule. Localization studies revealed the presence of both transporters in the plasma membrane, and expression in nodule cortex cells and vascular endodermis. Functional analysis in soybean showed that repression of GmUPS1-1 and GmUPS1-2 in nodules leads to an accumulation of ureides and decreased nitrogen partitioning to roots and shoot. It was further demonstrated that nodule development, nitrogen fixation and nodule metabolism were negatively affected in RNAi UPS1 plants. Together, we conclude that export of ureides from nodules is mediated by UPS1 proteins, and that activity of the transporters is not only essential for shoot nitrogen supply but also for nodule development and function.

Keywords: allantoin, allantoic acid, legume, nodule development, nitrogen fixation, metabolism, ureide export

INTRODUCTION

Soybean (Glycine max L. Merr.) is used as both a food source and a biofuel crop due to its high seed protein and oil levels, and globally its cultivation is exceeded only by wheat and maize (Stacey et al., 2004). Like other legumes, soybean plants are not dependent on nitrogen (N) fertilization for growth due to their ability to form symbioses with atmospheric di-nitrogen (N₂)-fixing bacteroids located in root nodules. While glutamine and asparagine are the main products of N₂ fixation in temperate legumes such as pea and Faba bean, in soybean and Phaseolus vulgaris nodules the ureides allantoin and allantoic acid are synthesized. These ureides are the primary transport form of nitrogen from nodules to the shoot (Rainbird, 1982; Smith and Atkins, 2002; Smith et al., 2002; Atkins and Smith, 2007).

For fixation, N₂ enters the bacteroids and is reduced to ammonia by a bacterial nitrogenase. Ammonia (or ammonium) is then released into the cytosol of the infected nodule cell via the peribacteroid membrane, and assimilated to glutamine (Morey et al., 2002; Obermeyer and Tyerman, 2005; Masalkar et al., 2010). In ureide-synthesizing legumes, glutamine moves into both mitochondria and plastids, where it is utilized for de novo purine synthesis (Smith and Atkins, 2002). Purines are rapidly degraded to xanthine, which is released to the cytosol and diffuses from infected to uninfected nodule cells. There, xanthine is oxidized in the cytosol to uric acid (Datta et al., 1991), which is then converted in the peroxisomes via the intermediates 5-hydroxyisourate and 2-oxo-4-hydroxy-4-carboxy-5-ureidoimidazoline to allantoin (Hanks et al., 1981; VandenBosch and Newcomb, 1986; Todd et al., 2006; Werner and Witte, 2011). Allantoic acid is produced in the endoplasmic reticulum from allantoin (Werner et al., 2008). Nodule ureide levels are dependent on the legume species (Atkins and Smith, 2007), and reach concentrations of 94 mm in soybean nodule exudate (Streeter, 1979). Following synthesis, the ureides are transported to the nodule vasculature, and leave in the xylem for shoot N supply. More than 80% of the N compounds that exit soybean nodules and that are translo-
cated in the transpiration stream may be in the form of ureides, and the allantoin to allantoic acid ratio may vary from 1:1 to 1:5, depending on the developmental stage of the plant (McClure and Israel, 1979; Streeter, 1979; Schubert, 1981; Rainbird et al., 1984; Gordon et al., 1985).

Soybeans develop spherical, determinate nodules with an inner region that contains the bacteroid-infected cells that function in N₂ fixation and N assimilation, as well as uninfected cells where ureide synthesis occurs. This central zone is surrounded by a layer of inner cortex cells, comprised of the distributing zone and boundary layer, that is bordered by a middle cortex, the sclerid layer, the outer cortex, and finally the periderm (Guineil, 2009). Vascular bundles encircled by an endodermis are located at the periphery adjacent to the inner cortex, and are connected with the root vascular system. Ureide transport within, and export out of, nodules has not been resolved, but may involve a symplasmic and apoplastic route (Brown et al., 1995). In the symplasmic pathway, following synthesis, the ureides travel from the uninfected cells via plasmodesmata to the inner cortex cells, the endodermis and then the nodule vasculature, where they are loaded into the xylem for translocation to the shoot (Selker, 1988; Walsh et al., 1989). Alternatively, ureides are released from the uninfected cells and move via the apoplast to the inner cortex or the vascular endodermis. In soybean, both the Casparian strip of the vascular endodermis (Pate et al., 1969; Walsh et al., 1989) and the boundary layer of the exterior-most inner cortex, where the intercellular wall spaces are occluded by glycoproteins (Parsons and Day, 1990; James et al., 1991; Webb and Sheehy, 1991; Brown and Walsh, 1994), block apoplastic flow of ureides to the xylem (Streeter, 1992). These barriers require uptake of apoplastic ureides into the inner cortex and endodermis cells, respectively, for export from nodules (Brown et al., 1995).

Recently, a French bean (P. vulgaris L.) protein called PvUPS1 (ureide permease 1) was identified that mediates transport of allantoic acid in yeast and is localized in the nodule endodermis (Péllissier et al., 2004). However, the physiological function of UPS1 transporters in legumes, let alone in nodules, has not been demonstrated. In addition, allantoic acid transporters have yet to be identified and characterized in planta. UPS transporters have also been found in non-ureide-transporting plant species, specifically AtUPS1–AtUPS5 in Arabidopsis (Desimone et al., 2002; Schmidt et al., 2004, 2006; Froissard et al., 2006). Heterologous expression in yeast and Xenopus laevis oocytes demonstrated that AtUPS1, 2 and 5 transport allantoic acid but have much higher affinities for purines and pyrimidines (e.g. xanthine and uracil), suggesting that compounds structurally related to allantoin represent the physiological substrates of UPS transporters in Arabidopsis.

The present work addresses the role of soybean UPS1 transporters in the nodules, and their significance in nodule physiology and development. We first describe the functional characterization of ureide permeases GmUPS1-1 and GmUPS1-2 in yeast, supporting a role for UPS1 proteins in allantoic as well as allantoic acid transport. Using cellular and subcellular localization studies, we then demonstrate that GmUPS1-1 and 1-2 are plasma membrane proteins that are expressed in the nodule inner cortex and vascular endodermis, suggesting a role in export of both allantoin and allantoic acid from the nodule. This is confirmed by phenotypic, molecular and biochemical analyses of composite soybean plants with silenced GmUPS1 expression in nodules. Supported by molecular, structural and physiological studies, it is further demonstrated that ureide transport processes are important for nodule development, and influence atmospheric N₂ fixation and N assimilation. Finally, the function of the two ureide transporters in N transfer from nodules to shoot is discussed, and their importance for nodule function is evaluated.

RESULTS

GmUPS1-1 and GmUPS1-2 function in ureide import into the soybean cells

Using an RT-PCR approach and primers designed along soybean homologs of PvUPS1 (Péllissier et al., 2004), we isolated two UPS1 cDNAs from soybean nodules that share 98 and 96% similarity at the nucleotide and amino acid level, respectively. The putative ureide transporter genes were named GmUPS1-1 (Glyma01g07120) and GmUPS1-2 (Glyma02g12970).

To determine whether the GmUPS1 proteins are functional transporters, direct uptake studies using [14C]allantoic acid and other substrates of the purine synthesis or salvage pathway in in addition to allantoin, competition experiments measuring [14C]allantoic acid uptake in the presence of a 10-fold molar excess of non-radioactive competitors were performed (Figure 1c). The results show that xanthine and uracil are strong competitors for allantoic acid uptake into yeast, similar to what was shown for French bean and Arabidopsis UPS1s (Desimone et al., 2002; Péllissier et al., 2004; Schmidt et al., 2004, 2006). However, while the allantoic acid level in nodules are high, in comparison the xanthine and uracil concentrations are negligible (Fujihara and Yamaguchi, 1978), and therefore they are most probably not substrates for the GmUPS1 transporters under physiological conditions. In contrast to previous studies, here we found that allantoic acid also
competed with allantoin uptake, indicating that GmUPS1-1 and GmUPS1-2 transport allantoic acid as well (Figure 1c). However, it may also be possible that allantoic acid partially inhibits the transport of allantoin without being transported itself.

The subcellular localization of UPS proteins is unknown, and to analyze whether the GmUPS1 proteins are functioning in cellular import or transport across subcellular membranes, GFP–GmUPS1-1 and GFP–GmUPS1-2 fusion proteins were localized in Nicotiana benthamiana leaf cells. Using confocal laser scanning microscopy, it was demonstrated that both GmUPS1-1 and GmUPS1-2 are targeted to the plasma membrane, which was even more evident when the leaves were plasmolyzed (Figure 2). Together, the results suggest a role for GmUPS1 proteins in cellular uptake of apoplastic allantoin and allantoic acid.

UPS1 transporters are expressed in the nodule cortex and vascular endodermis

To determine whether there are differences in the location of function between GmUPS1-1 and GmUPS1-2 in soybean nodules, RNA localization studies were performed. The in situ RT-PCR method was used, as, in contrast to the conventional RNA hybridization procedure, it allows specific amplification of highly similar genes and has fewer problems with background staining (Lee and Tegeder, 2004).

Taken together, our results differ from previous studies with French bean PvUPS1. In addition to expression in the endodermis and vascular bundles (Péllissier et al., 2004), both GmUPS1-1 and GmUPS1-2 were also expressed in the inner cortex (Figure 3a–f), suggesting a role for GmUPS1-1 and GmUPS1-2 in allantoin and allantoic acid uptake along the route from uninfected nodule cells to the vasculature. Some GmUPS1-1 and GmUPS1-2 expression was also found in the outer cortex as well as in the sclerid layer. In these cells, the GmUPS1 transporters may function in retrieval of ureides from the apoplast to prevent leakage into the soil and to redirect them in the symplast to the vasculature.

In previous studies, we isolated the PvUPS1 promoter from French bean (1255 bp, Figure S1), and we used this to create a PvUPS1 promoter–GUS construct in nodulated composite soybean plants to test whether the promoter targets gene expression in nodules to the same cell types in which the GmUPS1 transporters are expressed (Figure 3a–d) and to determine whether this promoter could be used
for an UPS1 silencing approach in nodules (see below). Developing transgenic hairy roots were infected with Bradyrhizobium japonicum to induce production of transgenic nodules. The nodules were analyzed using GUS assays, and the results showed staining throughout nodule development (Figure 3g). GUS staining was specifically found in the nodule cortex and vasculature and the vascular endodermis (Figure 3h), consistent with the localization of GmUPS1-1 and GmUPS1-2 in nodules (Figure 3a–h). These results demonstrate that the PvUPS1 promoter is well suited to silence GmUPS1 expression in nodules (see below).

**Repression of UPS1 expression in nodules causes reduced nodule development**

To determine the physiological function of the GmUPS1 transporters in nodules, we silenced GmPS1-1 and GmUPS1-2 expression using composite soybean plants, a strategy that has been successfully applied in recalcitrant soybean to analyze gene function in nodules or roots (Subramanian et al., 2004, 2006; Collier et al., 2005; Libault et al., 2009). In ex vitro composite legumes, transgenic nodulated roots can be produced in combination with a non-transgenic shoot (Collier et al., 2005). For targeted GmUPS1 repression, both GmUPS1-1 and GmUPS1-2 were concurrently repressed in soybean nodules using an RNAi approach under the control of the PvUPS1 promoter (Figures 3g,h and 4, and Figure S1). Composite plants expressing RNAi GFP were used as controls to ensure that potential changes in RNAi UPS1 nodules were not due to alterations in gene expression caused by induction of the RNAi machinery (Figure 4). When analyzing the transgenic nodules, an obvious difference in nodule development was observed (Figure 4a–d). Although the total number of nodules was unchanged in RNAi UPS1 plants (Figure 4e), the number of medium-sized (1.59–1.98 mm diameter) and large (>1.98 mm) nodules was significantly decreased by 60% compared to the RNAi control, and the amount of small nodules (<1.59 mm) was increased by 51% (Figure 4f), suggesting an arrest in development of RNAi UPS1 nodules.

UPS1 expression experiments were performed using RNA from medium-sized nodules to analyze whether the observed nodule phenotype coincides with decreased levels of GmUPS1 transcripts. The results showed 50 and 80% reductions in GmUPS1-1 and GmUPS1-2 expression, respectively (Figure 4g).

Repression of UPS1 expression leads to increased ureide levels in nodules and affects N translocation from nodule to shoot

Cellular and subcellular localization as well as the biochemical analyses in yeast suggest that both GmUPS1-1 and GmUPS1-2 import allantoin and allantoic acid into inner cortex and endodermis cells for export from the nodules and translocation to the shoot. To further resolve UPS1 function in planta, ureide levels were determined in RNAi UPS1 nodules of various sizes (Figure 5). Ureide levels were significantly increased in all nodules by 20–116%, with the highest increase occurring in large nodules (Figure 5a–c). In general, the ureides comprised approximately 30% allantoin and 70% allantoic acid. The elevation in total ureides was
due to an increase in both allantoin and allantoic acid, supporting a role for GmUPS1-1 and GmUPS1-2 in export of the two ureides from the nodule.

Ureides leave the nodule via the xylem that is connected to the root vasculature. Levels of ureides in RNAi UPS1 roots and xylem were examined to analyze whether N transport from nodules to roots and finally the shoot was altered. Allantoin and allantoic acid contributed approximately 30 and 70%, respectively, of the total ureide levels in both roots and xylem sap (Figure 6a,b). A significant decrease in allantoin as well as allantoic acid levels was detected in RNAi UPS1 roots compared to the RNAi control roots, leading to an overall reduction in total ureide

Figure 5. Total ureides, and allantoin and allantoic acid concentrations in RNAi UPS1 nodules. (a) Small nodules (<3.5 mm²). (b) Medium nodules (3.5–4.5 mm²). (c) Large nodules (>4.5 mm²). The results are representative of at least two independent experiments. Measurements are from three pools of nodules, each derived from at least two roots of six plants. Error bars show standard deviation, and asterisks indicate significant differences from the RNAi control (P < 0.02). Values above the columns indicate the percentage change compared to RNAi controls.

Figure 4. Analyses of nodule development and gene expression in RNAi UPS1 nodules. (a, c) Nodulated RNAi UPS1 roots. Scale bars = 1 cm (a) and 2 mm (c). (b, d) Nodulated RNAi control roots. Scale bars = 1 cm (b) and 2 mm (d). (e) Total nodule number in RNAi plants. (f) Development of small, medium and large transgenic nodules on roots of RNAi UPS1 and RNAi control plants. For (e) and (f), transgenic nodules were derived from 16 RNAi UPS1 and RNAi control plants, respectively. The results are representative of at least two independent experiments. Error bars show standard deviation. One-way analysis of variance (ANOVA) was used to determine statistical significance. Asterisks indicate significant differences from the RNAi control nodules (P < 0.001). Values above the columns indicate the percentage change compared to RNAi controls.

(g) Real-time PCR analysis of GmUPS1 transporters and genes involved in nitrogen export from symbiosomes and in nodule ureide synthesis. RNA from medium-sized nodules was used. Expression of the ureide transporter genes GmUPS1-1 (Glyma01g07120) and GmUPS1-2 (Glyma02g12970), and genes encoding Nod26 involved in ammonium translocation across the peribacteroid membrane (N-26), nodule uricase Nod35 (Ur9) and allantoinasases ALN1–4 (ALN1–ALN4) were analyzed. The expression levels were measured from three technical replicates relative to miR1596a, mi156b or miR1552d used as control genes (Kulcheski et al., 2010). Shown is the percent change in gene expression relative to miR1552d expression, determined from the 

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amounts of 45% (Figure 6a). In addition, ureide levels (i.e. allantoin and allantoic acid) in the xylem sap of RNAi UPS1 composite plants were significantly decreased by 31% compared to control plants (Figure 6b), further confirming that nodule to root to shoot translocation of ureides was altered due to UPS1 repression in nodules. A decrease in N partitioning from nodule to shoot was also evident when analyzing the shoot phenotype of the composite plants. It was found that the leaf size was reduced in RNAi UPS1 shoots, and the intercostal fields of leaves were yellowish while the leaf veins were green, which is consistent with N deficiency symptoms (Figure 6c,d).

**DISCUSSION**

**Soybean UPS1 proteins mediate transport of allantoin and allantoic acid**

Nodulated legumes such as soybean and French bean use ureides as the main long-distance transport form of nitrogen, and plasma membrane transporters have been hypothesized to be required for partitioning of allantoin and allantoic acid from nodules to shoot (Pelissier et al., 2004). Recent transport studies with yeast and Xenopus oocytes expressing UPS transporters from Arabidopsis and French
Soybeans produce determinate nodules with a center comprising infected and uninfected cells. This central tissue is surrounded by an inner, middle and outer cortex ensconced with the vascular system that allows import of shoot/phloem-derived metabolites and export of the ureides from the nodules via the xylem (Selker, 1988). Symplastic movement of the ureides out of the nodules is generally possible as plasmodesmata connect the various nodule cells, creating a symplasmic continuum from the uninfected cells to the vascular pericycle cells (Selker and Newcomb, 1985; Selker, 1988; Brown et al., 1995). However, the frequency of plasmodesmata varies between the cell types and appears to be relatively low between the inner cortex and vascular endodermis, and it has been suggested that the plasmodesmata may be involved in entry of large amounts of shoot/phloem-derived sucrose and other metabolites to the infected cells for \( N_2 \) fixation (Brown et al., 1995). Metabolite analyses of the cortical apoplast showing significant levels of ureides and low amounts of sucrose confirm that sucrose moves symplasmically into the infected nodule zone while ureides are transported apoplastically to the vasculature (Walsh et al., 1989; Streeter, 1992; Streeter and Salminen, 1993; Brown et al., 1995). Nevertheless, when the ureides reach the Casparian strip of the vascular endodermis (Pate et al., 1969; Walsh et al., 1989), plasma membrane transporters are required for their import into the symplasm to bypass the cell-wall blockade (Pelissier et al., 2004). In addition, apoplastic flow may be blocked in the inner cortex by the boundary layer, where intercellular wall spaces contain glycoprotein occlusions, requiring import of ureides into the cortex cells (Newcomb et al., 1989; Parsons and Day, 1990; James et al., 1991; Webb and Sheehy, 1991; Streeter, 1992). Localization of GmUPS1-1 and GmUPS1-2 in the plasma membrane and to the inner cortex and vascular endodermis, as well as their functional analysis in yeast, support the existence of an apoplastic transport mechanism for ureides within the nodules, and the function of both GmUPS1-1 and GmUPS1-2 in uptake of allantoin and allantoin repressed (Figures 5 and 6a,b). We have no clear explanation why, in contrast to the GmUPS1 proteins, PvUPS1 does not transport or mediate the uptake of allantoic acid (Pelissier et al., 2004), although it is present in relatively high amounts in French bean nodules (Alamillo et al., 2010). However, PvUPS1 function was only analyzed in yeast, and future in planta studies may resolve the complete substrate spectrum of the PvUPS1 transporter. In addition, as in soybean, French beans may have two UPS1 proteins, and there may be some variation in substrate specificity between these proteins. In soybean, GmUPS1-1 and GmUPS1-2 appear to be the product of gene duplication (Schmutz et al., 2010), and our data show that they have similar substrate specificity, localization and function in plants.

GmUPS1 function is essential for ureide export from nodules and for shoot N supply

Soybeans demonstrated that UPS proteins mediate transport of allantoin and other heterocyclic products of the purine synthesis and degradation pathway with varying substrate affinity. However, the UPS proteins were not involved in allantoic acid transport (Desimone et al., 2002; Pelissier et al., 2004; Schmidt et al., 2004, 2006; Pelissier and Tegeder, 2007), and their physiological function remained unresolved. The results presented here suggest that soybean UPS1 proteins transport both allantoin and allantoic acid. This is supported by yeast transport studies (Figure 1) as well as biochemical analyses of RNAi UPS1 soybean plants showing an accumulation of allantoin and allantoic acid in nodules and a decrease of these ureides in roots and xylem sap when GmUPS1-1 and GmUPS1-2 expression in nodules is repressed (Figures 5 and 6a,b). We have no clear explanation why, in contrast to the GmUPS1 proteins, PvUPS1 does not transport or mediate the uptake of allantoic acid (Pelissier et al., 2004), although it is present in relatively high amounts in French bean nodules (Alamillo et al., 2010). However, PvUPS1 function was only analyzed in yeast, and future in planta studies may resolve the complete substrate spectrum of the PvUPS1 transporter. In addition, as in soybean, French beans may have two UPS1 proteins, and there may be some variation in substrate specificity between these proteins. In soybean, GmUPS1-1 and GmUPS1-2 appear to be the product of gene duplication (Schmutz et al., 2010), and our data show that they have similar substrate specificity, localization and function in plants.

GmUPS1 function is essential for ureide export from nodules and for shoot N supply
toxic acid into the symplasm for export out of the nodule and N shoot supply. This is further supported by functional analyses of the GmUPS1 transporters using RNAi UPS1 soybean plants, showing an accumulation of allantoin and allantoic acid in nodules, a decrease of the ureides in roots and xylem sap, and N deficiency symptoms in leaves (Figures 5 and 6).

GmUPS1 function is important for nodule metabolism and nitrogen fixation

Inhibition of N₂ fixation has been studied extensively, and it has been suggested that ureide accumulation in soybean shoots (de Silva et al., 1996; Serraj et al., 1999b; Vadez and Sinclair, 2001) and in nodules (Vadez et al., 2000; de Beer et al., 2007; van Heerden et al., 2008; Vauclare et al., 2010) under drought or cold stress down-regulates nodule nitrogenase activity. This regulation of N₂ fixation may occur via feedback inhibition induced by elevated N amounts in the leaves, or it may be directly controlled by high ureide levels (or related N compounds) in the nodules (Sinclair and Serraj, 1995; Serraj et al., 1999b, 2001; Vadez and Sinclair, 2000, 2001). Recent work with drought-stressed soybean nodules suggests that a local rather than a systemic N signal regulates nitrogenase activity (King and Purcell, 2005; Ladera et al., 2007). Our studies showing increased allantoin and allantoic acid levels in RNAi UPS1 nodules and a concurrent decrease in N₂ fixation are in agreement with this (Figures 5 and 7a). Further, our results confirm previous predictions that the observed ureide accumulation in nodules and the subsequent effects on nodule activity are due to altered ureide export (Streeter, 1993, 2003; Walsh, 1995; Serraj et al., 1999a). Elevated levels of N₂ fixation products also appear to feedback-regulate N delivery from the symbiosome and N metabolism in nodules, as indicated by decreased transcript levels of UR9 and ureide synthesis genes (Figure 4g), corroborating the previously described regulation of primary nodule metabolism under drought (and high nodule N) conditions (Larrainzar et al., 2007).

GmUPS1 is critical for nodule development

Generally, successful nodulation and nodule development occurs under N-limiting conditions, and it is widely accepted that high N levels in the soil inhibit legume nodulation (Herridge et al., 1984) and nodule development (Imsande, 1986), in addition to their detrimental effect on nitrogenase activity (Purcell and Sinclair, 1990). In our studies, the number of nodules was not changed in RNAi UPS1 plants, but nodule development was inhibited (Figure 4a–f). In addition, independent of the nodule size, the infected cells were generally smaller in RNAi UPS1 plants compared to the control plants (Figure 7b), probably due to differences in bacteria infection, bacteroid development or endo-reduplication of the infected cells (Parsons and Day, 1990; James et al., 1991; Maunoury et al., 2008; Libault et al., 2011). Together, the results demonstrate that N transporter activity in nodules is not only essential for N export from the nodule, but also for regulating nodule N levels affecting nodule size, as well as the development and function of bacteroid-containing infected cells. They further indicate that a signal downstream of nodule initiation causes the observed alteration, and that the signal is nodule-localized rather than systemic (Eaglesham, 1989; Abaidoo et al., 1990). The high levels of ureides or related N compounds in RNAi UPS1 nodules may induce the developmental changes, as a similar effect was observed when nodules were supplied with nitrate, and nodule maturation resumed upon removal of N (Fujikake et al., 2003). However, inter-related metabolic pathways such as carbon metabolism are most probably also altered in RNAi UPS1 nodules, and may lead to the arrest in nodule growth (Parsons and Day, 1990; James et al., 1991; Green and Emerich, 1997; Van Dao et al., 2008; Libault et al., 2011). Future transcriptome and metabolome analyses may help to identify the signals as well as resolve further interactions between N transport and nodule physiology.

PERSPECTIVES

This work demonstrates that UPS1 transporters are important for the export of allantoin and allantoic acid out of the nodules. Our results further suggest that ureide transporters control nodule allantoin and allantoic acid levels, and that these ureides or related N compounds provide regulatory signals for atmospheric N₂ fixation and nodule metabolism, growth and potentially rhizobia infection. Investigations are now required to establish how nodule ureide transporters are controlled, and if and how allantoin or allantoic acid act as a signaling molecule for nodule function and development. We also intend to address whether increased ureide export positively affects N₂ fixation and N shoot supply in stressed environments (i.e. drought and cold) and non-stressed environments, with beneficial consequences for plant performance and seed yield.

EXPERIMENTAL PROCEDURES

Materials and growth conditions

Plants of Glycine max cultivar Hutcheson (Buss et al., 1988) were grown in the growth chamber under a 16 h photoperiod and light intensity of 1000 µmol photons m⁻² sec⁻¹. The day/night temperature and relative humidity were 26°C/21°C and 50%/70%, respectively. For DNA or RNA expression analyses, soybean plants were grown in Turface MVP Infield Conditioner (Hummert, http://www.invitrogen.com/) in 4 L pots. The seeds were inoculated with Bradyrhizobium japonicum strain USDA110. Plants were watered twice per day (approximately 1 L each time), and fertilized every 2 days using an N-free plant nutrient solution (Lullien et al., 1987). The production of nodulated composite soybean plants and their growth media is explained below. French bean (Phaseolus vulgaris cv. Redland) plants were grown in the greenhouse as previously described (Péllissier et al., 2004).
Nitrogen transport in soybean nodules

Isolation of soybean UPS1 transporters

To isolate the soybean UPS1 transporters, primers (Table S1) were designed based on the PvUPS1 homologs Glyma01g07120 (GmUPS1-1) and Glyma02g12700 (GmUPS1-2) [http://www.phytozome.net or the National Center of Biotechnology Information database (http://www.ncbi.nlm.nih.gov)]; accessions XP_003516366 and XP_003518768, respectively). Nodule cDNA was synthesized as previously described (Collier et al., 2005), and PCR was performed using Platinum® Taq high-fidelity DNA polymerase (Invitrogen, http://www.invitrogen.com/). PCR products were cloned into pGEM®-T-Easy (Promega, http://www.promega.com/) and sequenced.

Isolation of the PvUPS1 promoter

For promoter isolation, genomic DNA of Glyma01g07120 (GmUPS1-1) and Glyma02g12700 (GmUPS1-2) [http://www.phytozome.net or the National Center of Biotechnology Information database (http://www.ncbi.nlm.nih.gov/); accessions XP_003516366 and XP_003518768, respectively] was analyzed for regulatory motifs using the Plant Cis-Acting Element Database [PLACE] (Higo et al., 1998). Putative regulatory elements and references are shown in Figure S1.

Biochemical characterization of GmUPS1-1 and GmUPS1-2 in yeast

GmUPS1-1 and GmUPS1-2 cDNAs were transferred from pGEM®-T-Easy into the EcolI site of yeast expression vector pDR196 (Rentsch et al., 1995). Yeast strain dald/dald, which is deficient in allantoin transport (Desimone et al., 2002), was transformed with GmUPS1-1 or GmUPS1-2/dald/pDR196 as described by Dohmen et al. (1991), and growth complementation studies were performed on medium containing allantoin as sole nitrogen source. PvUPS1/pDR196 (Pélissier et al., 2004) and the empty vector were used as controls. Direct uptake studies using [7-14C]-labeled allantoin as well as competition studies to determine the substrate specificity of GmUPS1-1 and GmUPS1-2 were performed as previously described (Pélissier et al., 2004). Allantoin, allantoic acid and other substrates of the purine salvage or salvage pathway including xanthine, uric acid, uracil, hypoxanthine and glyoxylic acid were used as competitors and supplied in 10-fold excess.

Construct preparation for plant transformation

For construct preparation, vectors from the Modular Binary Construct System (MBCS, Collier et al., 2005) kindly provided by Dr Christopher Taylor (Department of Plant Pathology, Ohio State University, Wooster, OH, USA) were used. These vectors are modified pBluescript IIKS+ vectors containing a variety of promoters/genes and either a uidA intron followed by a nuclear localization signal (NLS) (Raikhel, 1992), the uidA gene (GUS) encoding β-glucuronidase (Jefferson et al., 1987), and the nopaline synthase (NOS) terminator (Bevan et al., 1983).

For subcellular localization, UPS1 transporters–GFP gene fusion constructs were prepared using an MBCS vector containing the SU promoter, the SU intron and a full-length GFP5 gene (Siemering et al., 1996). The Pad restriction sites were substituted by Sdal sites, and the GFP gene was replaced with either GmUPS1-1 or GmUPS1-2 (BarmlH/Sacl). An adapter (Table S1) was added using BamHI/BglII upstream of the UPS1 cDNAs. A GFP5 gene without a stop codon was cloned into the BamHI site upstream of the adapter, resulting in a final cassette comprising SU promoter–SU intron–GFP adapter–GmUPS1-1/GmUPS1-2-NOS terminator.

To prepare an RNAi-UPS1 construct, an MBCS vector containing the figwort mosaic virus (FMV) promoter, Kannibal intron (Hellwell et al., 2002) and octopine synthase terminator (OCS) was used. First, the FMV promoter was replaced using Sact/Kpnl by a PvUPS1 promoter PCR product tailored with Sact/Kpnl sites. An adapter containing Ndel and SaI restriction sites (see Table S1) was then cloned using Kpnl downstream of the UPS1 promoter. To simultaneously silence both GmUPS1 transporters in nodules, a PCR fragment was produced along a conserved region of GmUPS1-1 and GmUPS1-2 (positions 31–562) and restriction sites were added (5′, XbaI and Ndel; 3′, SaI and XbaI; see Table S1 for primers). The GmUPS1 silencer fragments were cloned using Ndel/SaI (sense) upstream of the Kannibal intron, and using XbaI (antisense) downstream of the intron, resulting in a final cassette comprising PV UPS1 promoter–UPS1 silencer (sense)–adapter–Kannibal intron–UPS1 silencer (antisense)–OCS terminator. To ensure that any phenotype observed in RNAi UPS1 plants is based on UPS1 repression and not an RNAi effect, a control vector was produced containing an exogenous (non-plant) gene, i.e. GFP. RNAi GFP constructs were created by cloning GFP silencer fragments (717 bp, see Table S1 for primers) instead of UPS1 silencers up stream and downstream of the Kannibal intron (Kpnl, sense; BarmlH/HindIII, antisense).

Depending on the construct (see above), the prepared cassettes were removed from the shuttle vectors using either Pad or Sdal, and transferred into the MBCS binary plasmid, which is a modified pBIN19 vector (Collier et al., 2005). The modified pBIN19 used for the RNAi constructs additionally contained an SU promoter–GUS–NOS terminator cassette on the T-DNA that allows visual identification of successful transformation of nodulated roots in composite plants using the GUS assay (see below). The binary vectors harboring the SU promoter–GFP–GmUPS1–1SU promoter–GFP–GmUPS1–2 constructs were transferred into Agrobacterium rhizogenes 18r12v (Veena and Taylor, 2007), a disarmed variant of strain NCPPB2659 (Combard et al., 1987), using electroporation (McCormac et al., 1998). Binary vectors containing the PV UPS1 promoter–GUS and the PV UPS1 promoter–RNAi UPS1 or RNAi GFP (control) constructs were transferred into strain NCPPB2659, that induces the development of (transgenic) hairy roots (Combard et al., 1987).

Subcellular localization of GmUPS1 proteins

For subcellular localization of GFP–GmUPS1–1 or GFP–GmUPS1–2 fusions, the Nicotiana benthamiana Domin leaf infiltration method was used (Sparkes et al., 2006). Agrobacterium rhizogenes 18r12v carrying GFP–GmUPS1–1 transporter fusion protein constructs was co-infiltrated with A. tumefaciens strain GV3101 pMP90 harboring the p19 protein gene of tomato bushy stunt virus to repress silencing in plant cells (Voinnet et al., 2003) and with A. rhizogenes 18r12v containing mCherry (Shaner et al., 2004) fused to aquaporin AP2P2A (Nelson et al., 2007; Arabidopsis Biological Resource Center stock number CD3-1007; http://www.arabidopsis.org) that has been shown to localize to the plant plasma membrane (Cutler et al., 2000). Leaf tissue was analyzed by confocal microscopy (Leica, http://www.leica.com/). Sodium chloride (1 M) was added to some specimens to induce plasmolysis to more clearly visualize localization of the GFP-GmUPS1 proteins to the plasma membrane.
GmUPS1-1 and GmUPS1-2 localization using in situ RT-PCR

For in situ RT-PCR localization experiments with GmUPS1-1 and GmUPS1-2, mature nodules of 35-day-old soybean plants were fixed in FAA (10% v/v formaldehyde, 5% v/v acetic acid and 50% v/v ethanol), dehydrated and paraffin-embedded as previously described (Lee and Tegeder, 2004). Nodule sections (10 μm) were prepared and used for in situ RT-PCR analysis (Lee and Tegeder, 2004). Two primer sets were used that specifically amplified GmUPS1-1 and GmUPS1-2, respectively (see Table S2). Amplification of 18S rRNA was used as a positive control, and negative controls were performed by omitting primers or Avian Myeloblastosis Virus (AMV) reverse transcriptase (Promega). The results were analyzed using light microscopy.

Production of ex vitro composite soybean plants and harvest of nodulated roots

Composite soybean plants expressing the PvUPS1 promoter–RNAi UPS1 or PvUPS1 promoter–RNAi GFP (control) constructs in nodulated roots were produced as described previously (Collier et al., 2005) but with some modifications to identify and remove non-transgenic roots at an early stage. In detail, decapitated 7-day-old soybean shoots were grown in Grodan cubes (Grodan, http://www.grodan.com/) inoculated with A. rhizogenes containing the specific constructs. After 2 weeks of growth, shoots with developed roots were removed from the cubes and adventitious roots were discarded, while putatively transgenic hairy roots originating from the teratoma region of the stem remained. The plants were then cultured in 50 ml polypropylene tubes with drainage holes (1 mm) containing Turface. After another 2 weeks of growth, individual roots of the RNAi plants were tested for successful transformation by using a lateral root for the GUS staining procedure (Jefferson et al., 1987). Amplification of 18S rRNA was used as a positive control, and negative controls were performed by omitting primers or Avian Myeloblastosis Virus (AMV) reverse transcriptase (Promega). The results were analyzed using light microscopy.

Acetylene reduction assay

Nitrogenase activity was determined using the acetylene reduction assay as described by House et al. (2004). Transgenic nodules were harvested from individual roots from at least 15 plants and grouped according to their size as described above. For the assay, 20 small, 10 medium or five large-sized nodules were placed at room temperature in 5 ml glass vials sealed with rubber septa (Chemglass, http://www.chemglass.com/). The headspace gas (250 μl) was replaced with acetylene, and a 250 μl gas sample was withdrawn after 20 min of incubation, and the ethylene produced was measured using a Shimadzu GC-8A gas chromatograph (Shimadzu, http://www.shimadzu.com/). Approximately 30 total measurements were performed for RNAi UPS1 or RNAi control nodules. Acetylene reduction to ethylene was calculated as previously described (House et al., 2004).

Structural analysis of nodules

Various sizes of nodules (described above) were fixed (4% glutaraldehyde, 2% paraformaldehyde, 50 mm PIPES buffer), embedded in London Resin White acrylic resin (Ted Pella, http://www.tedpella.com/), sectioned (1 μm) and stained with safranin as previously described (Fortin et al., 1987). Stained sections were imaged by light microscopy using a Leica DM LFS microscope equipped with a Leica DFC 300 FX cooled CCD camera.

Statistical analysis

Data are shown for one set of at least 16 RNAi UPS1 and RNAi control plants, respectively, but are representative of at least two independently grown sets of plants. Results are from approximately

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25–30 transgenic noduleated roots (including lateral roots), and are presented as means ± standard deviation. One-way analysis of variance (ANOVA) was used to determine statistical significance using SIGMASTAT 3.0 (Systat Software, http://www.systat.com/).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. PvUPS1 promoter analysis.
Table S1. Primers used for isolation of GmUPS1-1 and GmUPS1-2 and preparation of constructs.
Table S2. Primers used for GmUPS1-1 and GmUPS1-2 in situ RT-PCR localization studies and for expression analyses of genes involved in nitrogen metabolism and transport.

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