Report

Current Biology

Increasing Nitrogen Fixation and Seed Development in Soybean Requires Complex Adjustments of Nodule Nitrogen Metabolism and Partitioning Processes

Highlights

- Increasing nodule ureide export improves nitrogen fixation and shoot nutrition
- UPS1 function is coupled with nodule metabolic and transport pathways
- Nitrogen partitioning processes and nodulation are linked
- Organic nitrogen transporters can be used in plant breeding and seed production

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In Brief

Legumes access atmospheric nitrogen (N) in a symbiotic relationship with bacteria that reside in root nodules. Carter and Tegeder demonstrate that enhancing N export from nodules leads to improved N fixation, shoot nutrition, and seed yield. They show that N transport out of nodules is tightly linked to nodule metabolic and partitioning pathways.

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Increasing Nitrogen Fixation and Seed Development in Soybean Requires Complex Adjustments of Nodule Nitrogen Metabolism and Partitioning Processes

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SUMMARY

Legumes are able to access atmospheric di-nitrogen (N_2) through a symbiotic relationship with rhizobia that reside within root nodules. In soybean, following N_2 fixation by the bacteroids, ammonia is finally reduced in uninfected cells to allantoin and allantoic acid [1]. These ureides present the primary long-distance transport forms of nitrogen (N), and are exported from nodules via the xylem for shoot N supply. Transport of allantoin and allantoic acid out of nodules requires the function of ureide permeases (UPS1) located in cells adjacent to the vasculature [2, 3]. We expressed a common bean *UPS1* transporter in cortex and endodermis cells of soybean nodules and found that delivery of N from nodules to shoot, as well as seed set, was significantly increased. In addition, the number of transgenic nodules was increased and symbiotic N_2 fixation per nodule was elevated, indicating that transporter function in nodule N export is a limiting step in bacterial N acquisition. Further, the transgenic nodules showed considerable increases in nodule N assimilation, ureide synthesis, and metabolite levels. This suggests complex adjustments of nodule N metabolism and partitioning processes in support of symbiotic N_2 fixation. We propose that the transgenic *UPS1* plants display metabolic and allocation plasticity to overcome N_2 fixation and seed yield limitations. Overall, it is demonstrated that transporter function in N export from nodules is a key step for enhancing atmospheric N_2 fixation and nodule function and for improving shoot N nutrition and seed development in legumes.

RESULTS AND DISCUSSION

Expression of Common Bean *UPS1* in Soybean Nodules Leads to an Increase in Nodule N Export, Shoot N Nutrition, and Seed Number

In legume-rhizobia symbioses, bacteroids reside in root nodules and fix atmospheric N_2. The resulting ammonia (NH_3) is transported across the symbiosome membrane into the infected host cells for reduction to glutamine (Figure 1A) [7–9]. In soybean nodules, glutamine is channeled into the purine synthesis and degradation pathways, and finally uric acid is synthesized [1]. After symplasmic movement into uninfected cells, uric acid is used for allantoin synthesis, and allantoic acid is produced from allantoin [10, 11]. The ureides allantoin and allantoic acid are generally released into the apoplasm and move toward the vasculature located at the nodule periphery (Figures 1A and 1B) [12, 13]. Apoplasmic flow is finally blocked by the Casparian strip of the vascular endodermis and the boundary layer of the inner cortex [14–16]. These barriers require the ureides to be taken up into cortex or endodermis cells in order to continue their journey to the xylem (Figures 1A and 1B) [12, 17]. Recent studies have demonstrated that ureide import into these cell types involves membrane-localized ureide permeases (UPS1) [2, 3]. In the current study, we increased expression of ureide importers in cortex and endodermis cells to enhance N flow out of nodules and to examine the consequences for shoot N nutrition, sink development, and nodule function.

PvUPS1 ureide permease from common bean (*Phaseolus vulgaris*) [3, 6] was expressed in soybean nodules under control of the corresponding *PvUPS1* promoter [2]. When analyzing stable transgenic soybean plants expressing a *PvUPS1* promoter-β-glucuronidase (GUS) construct, it was shown that this promoter targets gene expression to nodule cortex and endodermis cells (Figure S1) [2]. Two transgenic soybean lines, *UPS1* overexpressors *UPS1*-OE1 and *UPS1*-OE2, were produced, and nodulated plants of the T4 and T5 generations were analyzed. First, *PvUPS1* expression studies were performed using nodules of these soybean lines, and results demonstrated the presence of *PvUPS1* transporter transcripts (Figure 1C). When analyzing the xylem sap of the *UPS1*-OE plants, a significant increase in xylem ureide levels by up to 73% was observed (Figure 1D). Both allantoin and allantoic acid were elevated, supporting that UPS1 transporters regulate the amount of ureides leaving the nodule (cf. [2, 3, 12]) and that their overexpression leads to improved shoot N supply. Further, the *UPS1*-OE1 and -OE2 plants seem to display an increase in the xylem allantoin-to-allantoic acid ratio compared to wild-type (Figure 1D), which is in line with the notion that PvUPS1 preferentially transports allantoin, as previously discussed [2, 3].

To determine the effects of the improved shoot N allocation in OE plants, we analyzed development of reproductive sink organs. The pod number was elevated between 14% and 41%, dependent on the *UPS1* overexpressor (Figure 1E). In

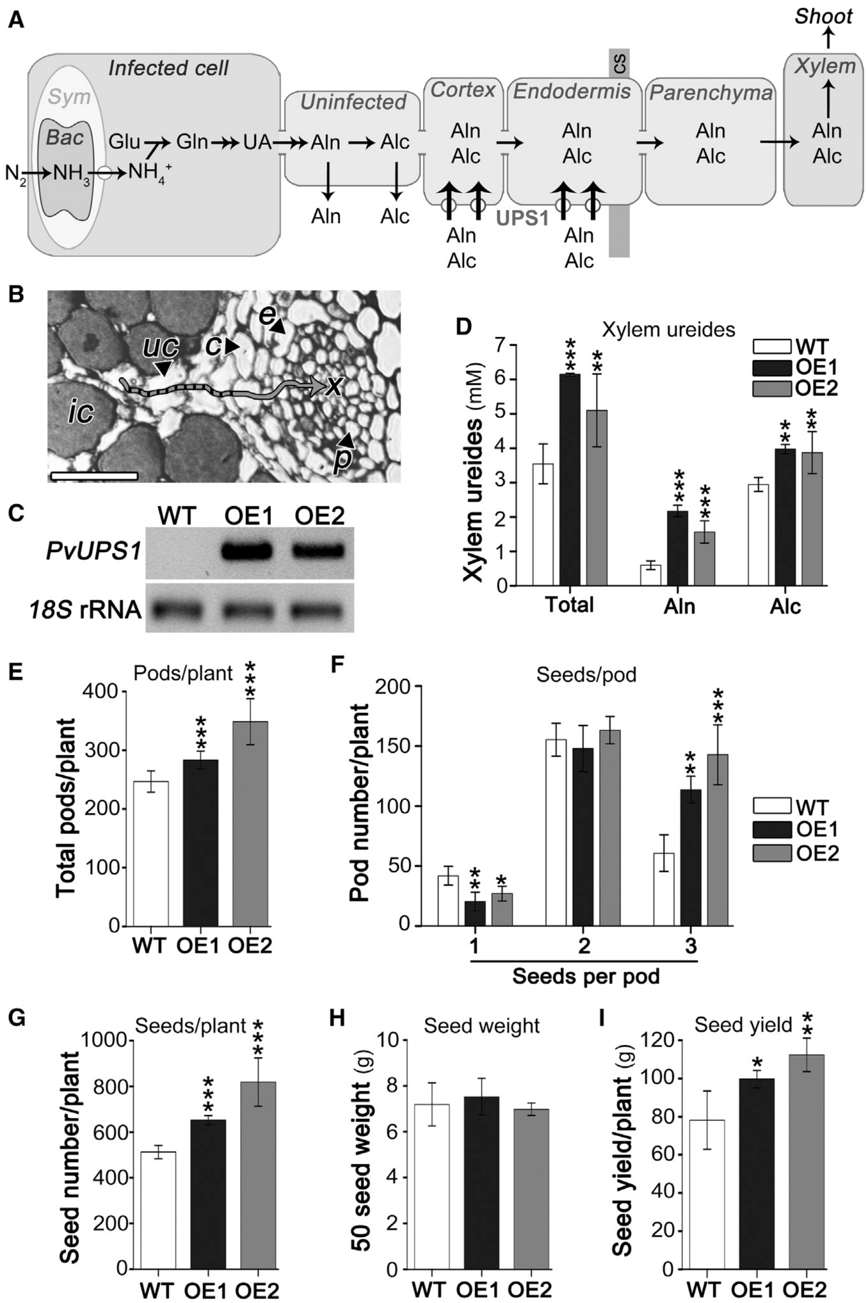

Figure 1. Effects of *UPS1* Overexpression in Soybean Nodules on Shoot Nitrogen Supply and Seed Development

(A) Model of N_2 fixation, N assimilation, and ureide (i.e., allantoin and allantoic acid) synthesis and transport in nodules of soybean *UPS1* overexpressors (OE1 and OE2) and wild-type (WT) plants. (Sym, symbiosome; Bac, bacteroid; Glu, glutamate; Gln, glutamine; UA, uric acid; Aln, allantoin; Alc, allantoic acid; CS, Casparian strip; UPS1, ureide permease 1.) In this study, and in addition to the endogenous *GmUPS1* transporters, common bean (*P. vulgaris*) *UPS1* was expressed in cortex and endodermis cells of soybean nodules using a transgenic approach. Arrows with circles indicate transporters.

(B) Cross-section of a soybean nodule embedded in resin and stained with toluidine blue. Arrow: the dashed portion indicates apoplasmic ureide flow; the solid portion shows symplasmic movement after cellular import via UPS1 transporters. Arrowheads point to specific cell types (ic, infected; uc, uninfected; c, cortex; e, endodermis cell; x, xylem). Scale bar, 50 μm.

(C) *PvUPS1* expression in nodules using RT-PCR and 18S rRNA as control [4].

(D) Total ureides, allantoin, and allantoic acid levels in xylem sap exudates [2, 5, 6] (n = 5 plants).

(E) Number of pods per plant (n = 6 plants).

(F) Distribution of one-, two-, and three-seeded pods (n = 6 plants).

(G) Total number of seeds (n = 6 plants).

(H) Fifty-seed weight (n = 6 plants with five measurements per plant).

(I) Seed yield (n = 6 plants).

Results presented here are from one growth set but are representative of a minimum of two independently grown sets of plants. The data are mean ± SD. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$. See also Figure S1.

addition, a significant shift from pods that developed one seed per pod to pods with three seeds was observed (Figure 1F). Together, these led to an increase in total seed number between 27% and 60% in *UPS1*-OE compared to wild-type plants (Figure 1G). Whereas the seed weight was not changed in OE plants (Figure 1H), the overall seed yield per plant was improved by up to 36% (Figure 1I). This demonstrates that increased N delivery to the shoot positively affected development of reproductive sinks, and that overexpression of *UPS1* in nodules presents an effective strategy for improving seed yield in soybean. However, future research will need to address whether such striking yield increases can be maintained under field conditions.

N_2 Fixation per Nodule and Nodulation Are Increased in *UPS1* Overexpressors

Seed yield in nodulated legume crops is directly linked to bacterial N_2 fixation, which seems to be regulated by the plant N status [18–20]. Because the soybean plants in this study were not supplied with any N fertilizer, increased N export from nodules and allocation to the shoot, as well as higher seed numbers, (Figure 1) point to improved N acquisition from the atmosphere. In fact, analysis of total elemental N levels demonstrated that the *UPS1*-OE plants fixed significantly more N than wild-type (Figure 2A). Whereas nodule sizes were unchanged in the transgenic plants (Figure S3), nodule numbers were strongly elevated (Figure 2B), leading to an increase in plant nodule biomass of up to 80% (Figure 2C). When calculating the N_2 fixation rate per nodule based on the plant N content, we found that it was significantly upregulated in OE nodules (Figure 2D). This was consistent with observed increases in the activity of nitrogenase, the key enzyme for N_2 fixation, in the transgenic nodules (Figure 2E). Comparison

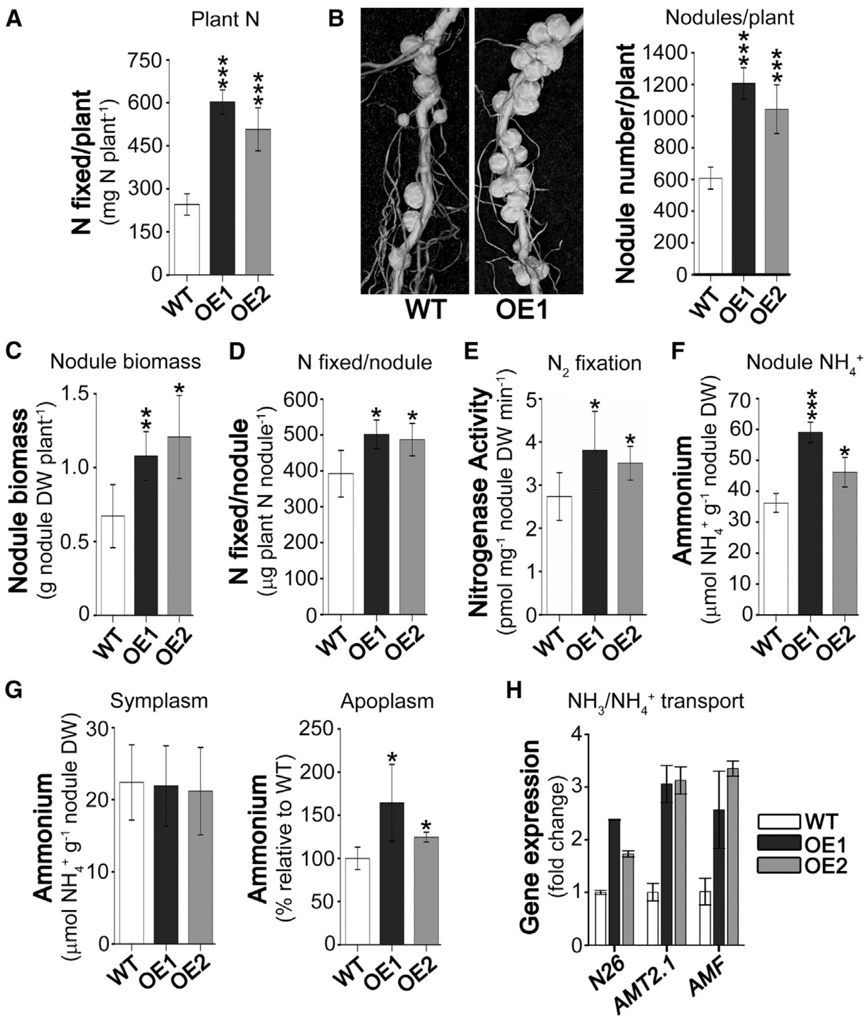

Figure 2. Analysis of Nodulation and Nitrogen Fixation in *UPS1*-OE Plants

(A) N fixed per plant based on the total amount of elemental N in shoot, roots, and nodules of *UPS1* overexpressors (OE1 and OE2) and WT plants [21] (n = 7 plants).

(B) Left: nodulated roots of *UPS1*-OE1 and WT plants. Right: nodule number (n = 6 plants).

(C) Nodule biomass (n = 6 plants). DW, dry weight.

(D) N fixed per nodule calculated from the total plant N content [21] and nodule number (n = 7 plants). See also Figure S2.

(E) Analysis of nodule nitrogenase activity using an acetylene reduction assay [22] (n = 7 plants; for each plant, three pools of ten nodules were measured).

(F and G) Analysis of NH_3/NH_4^+ levels in (F) whole nodules and (G) nodule symplasm and apoplasm (n = 4 pools of nodules; pools are from two plants each).

(H) Expression analysis of genes involved in NH_3/NH_4^+ transport using qPCR [23, 24]. Results are shown as fold change compared to WT, and are presented as the mean of three technical repetitions. For gene and primer information, see Table S1.

Results presented here are from one growth set but are representative of a minimum of two independently grown sets of plants. The data are mean ± SD. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$. See also Figures S2 and S3 and Table S1.

among nodule number and biomass (Figure 2C), and the N fixed per plant (Figure 2A) and nodule (Figure 2D), respectively, suggests that the impressive increase in N_2 fixation is, to a large extent, due to an increase in nodule initiation and development but also to an upregulation of nodule activity/metabolism (see also Figures 3 and 4). How the ectopic expression of the ureide transporter leads to the change in nodule development remains elusive but, based on the literature, it is reasonable to speculate that changes in N allocation to the *UPS1*-OE shoot, and a subsequent increase in sink development, may alter a systemic shoot-to-root signal controlling nodule number and potentially N_2 fixation [26–31].

Ammonia/Ammonium Levels and Partitioning Are Altered in *UPS1*-OE Nodules

In *UPS1*-OE nodules, NH_3/NH_4^+ amounts were significantly increased (Figure 2F), which relates to the observed higher N_2 fixation rates (Figures 2D and 2E; [7]; see above). On the other hand, increased levels of inorganic N compounds in nodules have been associated with N_2 fixation inhibition [20, 32–34]. Interestingly, when analyzing symplasmic versus apoplasmic nodule N concentrations [25], we found that cellular NH_3/NH_4^+ levels were not changed, whereas NH_3/NH_4^+ concentrations in the *UPS1*-OE nodule apoplasms were significantly increased (Figure 2G). This suggests that (1) NH_4^+ levels within the OE nodule cells are kept at steady state presumably to prevent NH_4^+ toxicity and N_2 fixation inhibition [35], and (2) excess NH_4^+ is temporarily sequestered in the acidic apoplasm by an ion-trapping mechanism [36], similar to what has been described for NH_4^+ storage in the vacuole [35, 37]. Upregulation of genes involved in reimport of NH_4^+ into the nodule cells (*AMT2.1* [38, 39]; *AMF* [40]; Figure 2H) supports that the inorganic N is finally recovered from the apoplasm for amino acid assimilation. Some of the NH_4^+ may also be retrieved for nodule export and subsequent reduction within the root, or for release into the environment [40, 41].

Amino Acid Metabolism Is Upregulated in *UPS1* Overexpressors

NH_3/NH_4^+ derived from the symbiosomes or retrieved from the apoplasm (see above) is assimilated into glutamine and other amino acids in nodule cells [42–44]. Expression analyses of genes involved in NH_3/NH_4^+ transport across the symbiosome (*N26* [45]) and host cell plasma membrane (AMT2.1 [39]) and in synthesis or deamination of the metabolically active amino acids glutamine (*GS1* [46]; *GS*2 [47]), glutamate (*GOGAT* [48]), aspartate (*AAT* [49]), and asparagine (*AS* [50]; *ASPG* [51]), showed increased transcript levels for all genes tested (Figure 3A). This

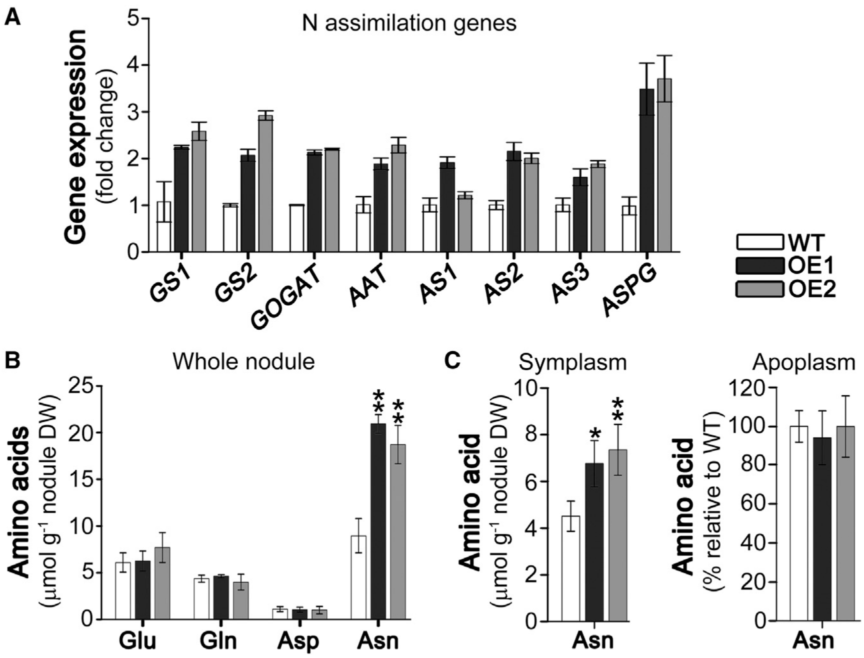

Figure 3. Amino Acid Biosynthesis and Asparagine Accumulation in *UPS1*-OE Nodules

(A) Expression of genes involved in N assimilation in *UPS1* overexpressors (OE1 and OE2) using qPCR [23, 24]. Results are shown as fold change compared to WT, and are presented as the mean of three technical repetitions. For gene and primer information, see Table S1.

(B and C) Amino acid levels in (B) whole nodules and (C) nodule symplasm and apoplasm (n = 4 pools of nodules; pools are from two plants each). Results presented here are from one growth set but are representative of a minimum of two independently grown sets of plants. The data are mean ± SD. $^{**}p < 0.01$, $^{*}p < 0.05$. See also Figure S2 and Table S1.

supports increased N allocation to, and an upregulation of, N metabolism in the transgenic nodules, which is in line with the observed increases in N_2 fixation.

When examining the corresponding amino acid concentrations in *UPS1*-OE versus wild-type nodules, we found that nodule asparagine amounts were increased due to higher levels of the amide in the symplasm, whereas levels of other initial amino acids were unchanged (Figures 3B and 3C). Generally, high levels of glutamine are thought to negatively feedback regulate N-assimilatory enzymes [52–54]. Thus, balancing the amount of glutamine may be essential for improved *UPS1*-OE nodule function and N assimilation. This is, for example, achieved by transfer of the amino N from glutamine to aspartate, resulting in elevated levels of asparagine (Figure 3). Further, an increased concentration of nodule aspartate has been shown to lead to a decline in N_2 fixation [20, 55]. Although the regulatory mechanism is not clear, it is fair to speculate that in the *UPS1*-OE nodules, aspartate is maintained at dynamic equilibrium through amination and deamination processes via aspartate aminotransferase and asparaginase, respectively, because the associated genes were upregulated (Figure 3A). In nodules, purines and finally ureides are also synthesized from aspartate, and transiently stored asparagine may be readily available for their synthesis via enzymatic conversion to aspartate [56]. In contrast to some reports that suggest a role of asparagine in N_2 fixation inhibition [55, 57–59], our work is in agreement with King and Purcell [20], demonstrating that N_2 fixation in soybean nodules occurs despite elevated asparagine amounts, and it supports that the amide serves in NH_4^+ detoxification and as a transient storage pool of N [60–62].

Ureide Metabolism Is Upregulated in *UPS1* Overexpressors

In soybean nodules, the majority of produced glutamine (and aspartate) is channeled into the purine synthesis pathway, and finally ureides are synthesized through purine degradation via uric acid [1, 63]. Expression of genes involved in purine (*PUR5* [64]; *ACP1* [65]) and ureide synthesis (*UR9* [66]; *HIUHase* [67]; *ALN1-4* [68]) was examined (see Figure S2 and Table S1), and results showed up to a 5-fold upregulation dependent on the gene and *UPS1*-OE line (Figure 4A).

There is plenty of debate about the relationship between ureide levels in nodules and N_2 fixation. A lot of evidence supports that accumulation of ureides in nodules is related to inhibition of N_2 fixation [2, 20, 34, 55], although recent work questions the role of ureides in the negative-feedback hypothesis [69]. Nevertheless, we were surprised to discover that upregulation of ureide export from nodules not only resulted in increased N_2 fixation and N assimilation but also in elevated total nodule ureide levels, including allantoin and allantoic acid (Figure 4B). However, in contrast to previous studies, we distinguished between symplasmic and apoplasmic ureide levels, and our biochemical analyses, together with the expression studies, support that synthesis of both ureides is increased in *UPS1*-OE nodules, resulting in enhanced allantoin and allantoic acid amounts in the symplasm (Figure 4C). In addition, and as previously reported [12], the levels of both ureides were generally higher in the symplasm versus apoplasm, thereby creating a concentration gradient for their passive movement into the cell-wall space. Whereas amounts of allantoic acid were also increased in the *UPS1*-OE nodule apoplasm, allantoin levels were not changed (Figure 4C). At apoplasmic pH, some allantoin is most probably hydrolyzed, and allantoic acid or ammonia is formed [70], contributing to the elevated levels of these two compounds in the extracellular compartment (see Figures 2G and 4C). It is further reasonable to assume that the continuous apoplasmic transformation of allantoin maintains a steep concentration gradient and promotes ongoing diffusive movement of this ureide from the nodule cells into the cell-wall space. Similarly, strong upregulation of allantoic acid production within the nodule cell via allantoinases (ALNs; Figure 4A) or spontaneous conversion of allantoin [70] seems to sustain a gradient for allantoic acid diffusion into the apoplasm.

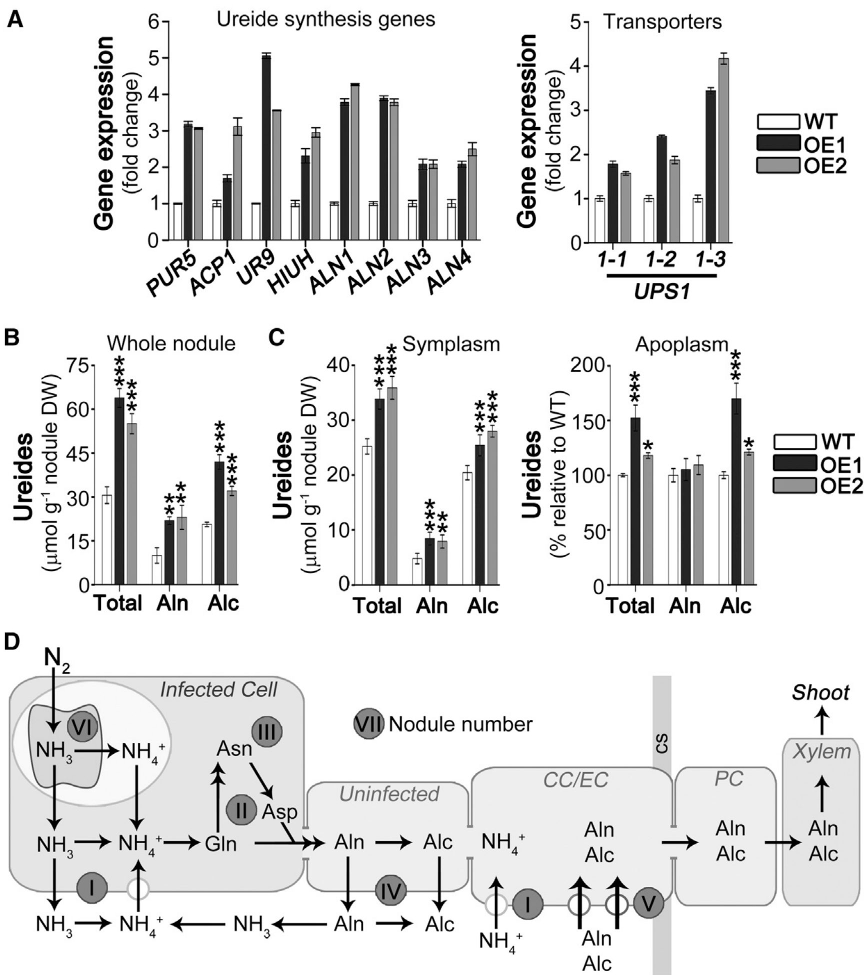

Figure 4. Analyses of Ureide Synthesis and Transport and Model of Nitrogen Metabolism and Partitioning Processes in *UPS1*-OE Nodules

(A) Expression of genes involved in ureide synthesis and transport using qPCR and nodule RNA from *UPS1* overexpressors (OE1 and OE2) and WT plants [23, 24]. Results are shown as fold change compared to WT, and are presented as the mean of three technical repetitions. For gene and primer information, see Table S1.

(B and C) Total ureides, allantoin, and allantoic acid levels of (B) whole nodules and (C) nodule symplasm and apoplasm [2, 5, 25] (n = 4 pools of nodules; pools are from two plants each).

Results presented here are from one growth set but are representative of a minimum of two independently grown sets of plants. The data are mean ± SD. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$.

(D) Model of key regulatory steps in *UPS1*-OE soybean nodules suggesting metabolic and allocation plasticity in support of increased N_2 fixation and nodule-to-shoot N supply. Seven (I–VII) regulatory events downstream of bacteroid function are proposed and discussed. (I) Ammonium levels within the host cells are kept at steady state to prevent potential NH_4^+ toxicity and N_2 fixation inhibition. (II) Glutamine and aspartate levels are kept constant to avoid downregulation of N_2 fixation and N assimilation. (III) Excess organic N is transiently stored as asparagine. (IV) Symplasmic and apoplasmic levels of allantoin and allantoic acid are adjusted to facilitate increased ureide export from nodules and to potentially prevent downregulation of N_2 fixation. (V) Movement of ureides from the apoplasm of uninfected cells toward the vascular bundles is controlled by UPS1 plasma membrane transporters located in cortex and endodermis cells. (VI and VII) The increased N demand of *UPS1*-OE shoots is met by both (VI) elevated N_2 fixation per nodule and (VII) an increase in nodule number. Arrows with circles indicate transporters. CC, cortex cell; EC, endodermis cell; PC, parenchyma cell; Asn, asparagine; Asp, aspartate.

See also Figure S2 and Table S1.

Endogenous Ureide Transport Processes Are Upregulated in *UPS1* Overexpressors

In previous work, we localized two soybean *UPS1* transporters to nodule cortex and endodermis cells (*GmUPS1-1* and *GmUPS1-2*) [2]. Using the soybean genome database Phytozome (https://phytozome.jgi.doe.gov/pz/portal.html), a third transporter (*GmUPS1-3*; Glyma01 g12980) was identified. Expression analysis of the endogenous transporters in *UPS1*-OE nodules showed that transcript levels of all three soybean *UPS1* transporters were upregulated (Figure 4A). Previous functional transporter analyses in yeast suggested that the soybean GmUPS1 proteins transport both ureides [2] whereas common bean PvUPS1 seems to preferentially transport allantoin [3], although the present work does not exclude that the latter may use both allantoin and allantoic acid as substrates. Nevertheless, the expression studies (Figure 4A), together with the increased nodule-to-shoot ureide allocation (Figure 1D), support that, besides PvUPS1, endogenous UPS1 transporters contribute to the accelerated ureide transport out of the transgenic soybean nodules. Further, the retrieval of allantoin and allantoic acid from the apoplasm promotes a concentration gradient allowing passive movement of the ureides from their location of synthesis toward the vascular bundles (see above). Moreover, the gradient between symplasmic and apoplasmic ureides is probably higher in *UPS1*-OE versus wild-type nodules due to increased UPS1 activity in cortex and endodermis cells. To resolve the actual concentration differences, rather than analyzing whole nodules, symplasmic and apoplasmic ureide concentrations would need to be determined for cells of the central zone versus cells adjacent to the nodule vasculature, for example by using metabolite sensors [71]. Nonetheless, in the *UPS1*-OE nodules, critical allantoin and/or allantoic acid accumulation, and related N_2 fixation inhibition (see above), seems to be prevented through the continuous and enhanced flow of ureides out of the nodule. Our work recommends that future research addressing the negative-feedback hypothesis will need to differentiate between allantoin and allantoic acid, and their location of accumulation, to clearly understand the regulatory role of nodule ureides in N_2 fixation. We also want to point out that it is still an open question whether symbiotic N_2 fixation inhibition is caused by a local/

nodule trigger, a systemic signal deriving from the shoot, or a combination of the two [20, 69, 72].

Conclusions

Overall, it is shown that enhancing N export from soybean nodules leads to increased N_2 fixation and nodule metabolism and promotes shoot N nutrition and seed development. Our work helps to draw a more complete, yet complex, picture of nodule physiology. We developed a model based on *UPS1* ureide transporter overexpression showing multilayered regulation of metabolic and transport events downstream of bacteroid function (Figure 4D). Finally, it can be concluded that a fundamental understanding of N metabolism and partitioning processes could clearly result in novel strategies to alter symbiotic N_2 fixation with the final goal of increasing legume productivity.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, three figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2016.06.003.

AUTHOR CONTRIBUTIONS

Conceived, M.T.; designed the experiments, M.T. and A.M.C.; performed the experiments, A.M.C.; analyzed the data, M.T. and A.M.C.; contributed reagents/materials/analysis tools, M.T.; wrote the paper, M.T. and A.M.C.

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REFERENCES

1. Smith, P.M.C., and Atkins, C.A. (2002). Purine biosynthesis. Big in cell division, even bigger in nitrogen assimilation. Plant Physiol. *128*, 793–802.
2. Collier, R., and Tegeder, M. (2012). Soybean ureide transporters play a critical role in nodule development, function and nitrogen export. Plant J. *72*, 355–367.
3. Pélissier, H.C., Frerich, A., Desimone, M., Schumacher, K., and Tegeder, M. (2004). PvUPS1, an allantoin transporter in nodulated roots of French bean. Plant Physiol. *134*, 664–675.
4. Santiago, J.P., and Tegeder, M. (2016). Connecting source with sink: the role of *Arabidopsis* AAP8 in phloem loading of amino acids. Plant Physiol. *171*, 508–521.
5. Vogels, G.D., and Van der Drift, C. (1970). Differential analyses of glyoxylate derivatives. Anal. Biochem. *33*, 143–157.
6. Pélissier, H.C., and Tegeder, M. (2007). PvUPS1 plays a role in source–sink transport of allantoin in French bean (*Phaseolus vulgaris*). Funct. Plant Biol. *34*, 282–291.
7. Bergersen, F.J. (1965). Ammonia? An early stable product of nitrogen fixation by soybean root nodules. Aust. J. Biol. Sci. *18*, 1–9.
8. Niemietz, C.M., and Tyerman, S.D. (2000). Channel-mediated permeation of ammonia gas through the peribacteroid membrane of soybean nodules. FEBS Lett. *465*, 110–114.
9. Lodwig, E.M., Hosie, A.H., Bourdès, A., Findlay, K., Allaway, D., Karunakaran, R., Downie, J.A., and Poole, P.S. (2003). Amino-acid cycling drives nitrogen fixation in the legume–*Rhizobium* symbiosis. Nature *422*, 722–726.
10. Todd, C.D., Tipton, P.A., Blevins, D.G., Piedras, P., Pineda, M., and Polacco, J.C. (2006). Update on ureide degradation in legumes. J. Exp. Bot. *57*, 5–12.
11. Werner, A.K., and Witte, C.P. (2011). The biochemistry of nitrogen mobilization: purine ring catabolism. Trends Plant Sci. *16*, 381–387.
12. Streeter, J.G., and Salminen, S.O. (1993). Alterations in apoplastic and total solute concentrations in soybean nodules resulting from treatments known to affect gas diffusion. J. Exp. Bot. *44*, 821–828.
13. Brown, S.M., Oparka, K.J., Sprent, J.I., and Walsh, K.B. (1995). Symplastic transport in soybean root nodules. Soil Biol. Biochem. *27*, 387–399.
14. Pate, J.S., Gunning, B.E.S., and Briarty, L.G. (1969). Ultrastructure and functioning of the transport system of the leguminous root nodule. Planta *85*, 11–34.
15. James, E.K., Sprent, J.I., Minchin, F.R., and Brewin, N.J. (1991). Intercellular location of glycoprotein in soybean nodules: effect of altered rhizosphere oxygen concentration. Plant Cell Environ. *14*, 467–476.
16. Brown, S.M., and Walsh, K.B. (1994). Anatomy of the legume nodule cortex with respect to nodule permeability. Funct. Plant Biol. *21*, 49–68.
17. Tegeder, M. (2014). Transporters involved in source to sink partitioning of amino acids and ureides: opportunities for crop improvement. J. Exp. Bot. *65*, 1865–1878.
18. González, E.M., Gordon, A.J., James, C.L., and Aresse-Igor, C. (1995). The role of sucrose synthase in the response of soybean nodules to drought. J. Exp. Bot. *46*, 1515–1523.
19. Parsons, R., and Sunley, R.J. (2001). Nitrogen nutrition and the role of root-shoot nitrogen signalling particularly in symbiotic systems. J. Exp. Bot. *52*, 435–443.
20. King, C.A., and Purcell, L.C. (2005). Inhibition of N_2 fixation in soybean is associated with elevated ureides and amino acids. Plant Physiol. *137*, 1389–1396.
21. Sanders, A., Collier, R., Trethewy, A., Gould, G., Sieker, R., and Tegeder, M. (2009). AAP1 regulates import of amino acids into developing *Arabidopsis* embryos. Plant J. *59*, 540–552.
22. House, B.L., Mortimer, M.W., and Kahn, M.L. (2004). New recombination methods for *Sinorhizobium meliloti* genetics. Appl. Environ. Microbiol. *70*, 2806–2815.
23. Kulcheski, F.R., Marcelino-Guimaraes, F.C., Nepomuceno, A.L., Abdelnoor, R.V., and Margis, R. (2010). The use of microRNAs as reference genes for quantitative polymerase chain reaction in soybean. Anal. Biochem. *406*, 185–192.
24. Livak, K.J., and Schmittgen, T.D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(−Delta Delta C(T)) method. Methods *25*, 402–408.
25. Streeter, J.G. (1992). Analysis of apoplastic solutes in the cortex of soybean nodules. Physiol. Plant. *84*, 584–592.
26. Carroll, B.J., McNeil, D.L., and Gresshoff, P.M. (1985). Isolation and properties of soybean [*Glycine max* (L.) Merr.] mutants that nodulate in the presence of high nitrate concentrations. Proc. Natl. Acad. Sci. USA *82*, 4162–4166.
27. Parsons, R., Stanforth, A., Raven, A.J., and Sprent, J.I. (1993). Nodule growth and activity may be regulated by a feedback mechanism involving phloem nitrogen. Plant Cell Environ. *16*, 125–136.
28. Searle, I.R., Men, A.E., Laniya, T.S., Buzas, D.M., Iturbe-Ormaetxe, I., Carroll, B.J., and Gresshoff, P.M. (2003). Long-distance signaling in nodulation directed by a CLAVATA1-like receptor kinase. Science *299*, 109–112.

29. Miyahara, A., Hirani, T.A., Oakes, M., Kereszt, A., Kobe, B., Djordjevic, M.A., and Gresshoff, P.M. (2008). Soybean nodule autoregulation receptor kinase phosphorylates two kinase-associated protein phosphatases in vitro. J. Biol. Chem. *283*, 25381–25391.

30. Magori, S., and Kawaguchi, M. (2009). Long-distance control of nodulation: molecules and models. Mol. Cells *27*, 129–134.

31. Tabata, R., Sumida, K., Yoshii, T., Ohyama, K., Shinohara, H., and Matsubayashi, Y. (2014). Perception of root-derived peptides by shoot LRR-RKs mediates systemic N-demand signaling. Science *346*, 343–346.

32. Purcell, L.C., and Sinclair, T.R. (1990). Nitrogenase activity and nodule gas permeability response to rhizospheric NH_3 in soybean. Plant Physiol. *92*, 268–272.

33. Schulze, J. (2004). How are nitrogen fixation rates regulated in legumes? J. Plant Nutr. Soil Sci. *167*, 125–137.

34. Ladrera, R., Marino, D., Larrainzar, E., González, E.M., and Arrese-Igor, C. (2007). Reduced carbon availability to bacteroids and elevated ureides in nodules, but not in shoots, are involved in the nitrogen fixation response to early drought in soybean. Plant Physiol. *145*, 539–5.

35. Britto, D.T., Siddiqi, M.Y., Glass, A.D., and Kronzucker, H.J. (2001). Futile transmembrane NH_4^+ cycling: a cellular hypothesis to explain ammonium toxicity in plants. Proc. Natl. Acad. Sci. USA *98*, 4255–4258.

36. Wood, C.C., Porée, F., Dreyer, I., Koehler, G.J., and Udvardi, M.K. (2006). Mechanisms of ammonium transport, accumulation, and retention in ooyctes and yeast cells expressing *Arabidopsis* AtAMT1;1. FEBS Lett. *580*, 3931–3936.

37. Loqué, D., Ludewig, U., Yuan, L., and von Wirén, N. (2005). Tonoplast intrinsic proteins AtTIP2;1 and AtTIP2;3 facilitate NH_3 transport into the vacuole. Plant Physiol. *137*, 671–680.

38. Udvardi, M., and Poole, P.S. (2013). Transport and metabolism in legume-rhizobia symbioses. Annu. Rev. Plant Biol. *64*, 781–805.

39. Simon-Rosin, U., Wood, C., and Udvardi, M.K. (2003). Molecular and cellular characterisation of LjAMT2;1, an ammonium transporter from the model legume *Lotus japonicus*. Plant Mol. Biol. *51*, 99–108.

40. Chiasson, D.M., Loughlin, P.C., Mazurkiewicz, D., Mohammadidehcheshmeh, M., Fedorova, E.E., Okamoto, M., McLean, E., Glass, A.D., Smith, S.E., Bisseling, T., et al. (2014). Soybean *SAT1* (*Symbiotic Ammonium Transporter 1*) encodes a bHLH transcription factor involved in nodule growth and NH_4^+ transport. Proc. Natl. Acad. Sci. USA *111*, 4814–4819.

41. Silvester, W.B., Parsons, R., and Watt, P.W. (1996). Direct measurement of release and assimilation of ammonia in the *Gunnera–Nostoc* symbiosis. New Phytol. *132*, 617–625.

42. Masalkar, P., Wallace, I.S., Hwang, J.H., and Roberts, D.M. (2010). Interaction of cytosolic glutamine synthetase of soybean root nodules with the C-terminal domain of the symbiosome membrane nodulin 26 aquaglyceroporin. J. Biol. Chem. *285*, 23880–23888.

43. Atkins, C.A. (1991). Ammonia assimilation and export of nitrogen from the legume nodule. In Biology and Biochemistry of Nitrogen Fixation, M.J. Dilworth, and A.R. Glenn, eds. (Elsevier Science), pp. 293–319.

44. Cullimore, J.V., and Bennett, M.J. (1988). The molecular biology and biochemistry of plant glutamine synthetase from nodules of *Phaseolus vulgaris* L. and other legumes. J. Plant Physiol. *132*, 387–393.

45. Fortin, M.G., Morrison, N.A., and Verma, D.P.S. (1987). Nodulin-26, a peribacteroid membrane nodulin is expressed independently of the development of the peribacteroid compartment. Nucleic Acids Res. *15*, 813–824.

46. Morey, K.J., Ortega, J.L., and Sengupta-Gopalan, C. (2002). Cytosolic glutamine synthetase in soybean is encoded by a multigene family, and the members are regulated in an organ-specific and developmental manner. Plant Physiol. *128*, 182–193.

47. Harrison, J., Pou de Crescenzo, M.A., Sené, O., and Hirel, B. (2003). Does lowering glutamine synthetase activity in nodules modify nitrogen metabolism and growth of *Lotus japonicus*? Plant Physiol. *133*, 253–262.

48. O'Rourke, J.A., Bolon, Y.T., Bucciarelli, B., and Vance, C.P. (2014). Legume genomics: understanding biology through DNA and RNA sequencing. Ann. Bot. *113*, 1107–1120.

49. Wadsworth, G.J. (1997). The plant aspartate aminotransferase gene family. Physiol. Plant. *100*, 998–1006.

50. Hughes, C.A., Beard, H.S., and Matthews, B.F. (1997). Molecular cloning and expression of two cDNAs encoding asparagine synthetase in soybean. Plant Mol. Biol. *33*, 301–311.

51. Pandurangan, S., Pajak, A., Molnar, S.J., Cober, E.R., Dhaubhadel, S., Hernández-Sebastià, C., Kaiser, W.M., Nelson, R.L., Huber, S.C., and Marsolais, F. (2012). Relationship between asparagine metabolism and protein concentration in soybean seed. J. Exp. Bot. *63*, 3173–3184.

52. Neo, H.H., and Layzell, D.B. (1997). Phloem glutamine and the regulation of O_2 diffusion in legume nodules. Plant Physiol. *113*, 259–267.

53. Glass, A.D.M., Britto, D.T., Kaiser, B.N., Kinghorn, J.R., Kronzucker, H.J., Kumar, A., Okamoto, M., Rawat, S., Siddiqi, M.Y., Unkles, S.E., and Vidmar, J.J. (2002). The regulation of nitrate and ammonium transport systems in plants. J. Exp. Bot. *53*, 855–864.

54. Rawat, S.R., Silim, S.N., Kronzucker, H.J., Siddiqi, M.Y., and Glass, A.D. (1999). AtAMT1 gene expression and NH_4^+ uptake in roots of *Arabidopsis thaliana*: evidence for regulation by root glutamine levels. Plant J. *19*, 143–152.

55. Vadez, V., Sinclair, T.R., and Serraj, R. (2000). Asparagine and ureide accumulation in nodules and shoots as feedback inhibitors of N_2 fixation in soybean. Physiol. Plant. *110*, 215–223.

56. Streeter, J.G. (1977). Asparaginase and asparagine transaminase in soybean leaves and root nodules. Plant Physiol. *60*, 235–239.

57. Serraj, R., Vadez, V., Denison, R.F., and Sinclair, T.R. (1999). Involvement of ureides in nitrogen fixation inhibition in soybean. Plant Physiol. *119*, 289–296.

58. Sulieman, S., Fischinger, S.A., Gresshoff, P.M., and Schulze, J. (2010). Asparagine as a major factor in the N-feedback regulation of N_2 fixation in *Medicago truncatula*. Physiol. Plant. *140*, 21–31.

59. Nasr Esfahani, M., Sulieman, S., Schulze, J., Yamaguchi-Shinozaki, K., Shinozaki, K., and Tran, L.S.P. (2014). Mechanisms of physiological adjustment of N_2 fixation in *Cicer arietinum* L. (chickpea) during early stages of water deficit: single or multi-factor controls. Plant J. *79*, 964–980.

60. Bauer, A., Joy, K.W., and Urquhart, A.A. (1977). Amino acid metabolism of pea leaves: labeling studies on utilization of amides. Plant Physiol. *59*, 920–924.

61. Givan, C.V. (1979). Metabolic detoxification of ammonia in tissues of higher plants. Phytochemistry *18*, 375–382.

62. Stewart, C.R. (1979). The effect of ammonium, glutamine, methionine sulfoximine and azaserine on asparagine synthesis in soybean leaves. Plant Sci. Lett. *14*, 269–273.

63. Shelp, B.J., Atkins, C.A., Storer, P.J., and Canvin, D.T. (1983). Cellular and subcellular organization of pathways of ammonia assimilation and ureide synthesis in nodules of cowpea (*Vigna unguiculata* L. Walp.). Arch. Biochem. Biophys. *224*, 429–441.

64. Smith, P.M.C., Mann, A.J., Goggin, D.E., and Atkins, C.A. (1998). AIR synthetase in cowpea nodules: a single gene product targeted to two organelles? Plant Mol. Biol. *36*, 811–820.

65. Penheiter, A.R., Duff, S.M.G., and Sarath, G. (1997). Soybean root nodule acid phosphatase. Plant Physiol. *114*, 597–604.

66. Suzuki, H., and Verma, D.P.S. (1991). Soybean nodule-specific uricase (Nodulin-35) is expressed and assembled into a functional tetrameric holoenzyme in *Escherichia coli*. Plant Physiol. *95*, 384–389.

67. Raychaudhuri, A., and Tipton, P.A. (2002). Cloning and expression of the gene for soybean hydroxyisourate hydrolase. Localization and implications for function and mechanism. Plant Physiol. *130*, 2061–2068.

68. Duran, V.A., and Todd, C.D. (2012). Four allantoinase genes are expressed in nitrogen-fixing soybean. Plant Physiol. Biochem. *54*, 149–155.

69. Gil-Quintana, E., Larrainzar, E., Seminario, A., Díaz-Leal, J.L., Alamillo, J.M., Pineda, M., Arrese-Igor, C., Wienkoop, S., and González, E.M. (2013). Local inhibition of nitrogen fixation and nodule metabolism in drought-stressed soybean. J. Exp. Bot. *64*, 2171–2182.
70. Vogels, G.D., de Windt, F.E., and Bassie, W. (1969). Hydrolysis and racemization of allantoin. Recl. Trav. Chim. Pays-Bas *88*, 940–950.
71. Jones, A.M., Grossmann, G., Danielson, J.Å., Sosso, D., Chen, L.Q., Ho, C.H., and Frommer, W.B. (2013). In vivo biochemistry: applications for small molecule biosensors in plant biology. Curr. Opin. Plant Biol. *16*, 389–395.
72. Marino, D., Frendo, P., Ladrera, R., Zabalza, A., Puppo, A., Arrese-Igor, C., and González, E.M. (2007). Nitrogen fixation control under drought stress. Localized or systemic? Plant Physiol. *143*, 1968–1974.