Soybean SAT1 (Symbiotic Ammonium Transporter 1) encodes a bHLH transcription factor involved in nodule growth and NH₄⁺ transport

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Glycine max symbiotic ammonium transporter 1 was first documented as a putative ammonium (NH₄⁺) channel localized to the symbiosome membrane of soybean root nodules. We show that Glycine max symbiotic ammonium transporter 1 is actually a membrane-localized basic helix–loop–helix (bHLH) DNA-binding transcription factor now renamed Glycine max bHLH membrane 1 (GmbHLHm1). In yeast, GmbHLHm1 enters the nucleus and transcriptionally activates a unique plasma membrane NH₄⁺ channel Saccharomyces cerevisiae ammonium facilitator 1. Ammonium facilitator 1 is a monovalent cation transport protein common to both yeast and plants. The activity of GmbHLHm1 in soybean nodules indicates an important role in nodule development and growth that is linked ultimately to an effective N₂-fixing symbiosis.

Results

GmbHLHm1 is a Membrane-Localized DNA-Binding bHLH TF. Sequence analysis shows that GmbHLHm1 belongs to a subset of the superfamily of plant bHLH TFs (5). GmbHLHm1-like proteins are predicted to be 28–42 kDa, contain a conserved bHLH DNA-binding domain, and a predicted C-terminal TD (SI Appendix, Fig. S1). The bHLH domain contains a conserved H-E-R amino acid motif.

Significance

The legume/rhizobia symbiosis involves a root-based exchange of bacterial fixed nitrogen for plant-derived photosynthetic carbon. The exchange takes place within the legume root nodule, which is a specialized root tissue that develops in response to plant and bacterial signal exchange. The bacteria reside within plant cells inside the nodule. In this study, we explore the activity of a membrane-bound soybean transcription factor, Glycine max basic-helix-loop–helix membrane 1, which is important for soybean nodule growth and is linked to the activity of a unique class of ammonium channels and to signaling cascades influencing a nodule circadian clock.


The authors declare no conflict of interest.

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Legumes can form symbiotic interactions with soil-borne N₂-fixing rhizobium bacteria. The symbiosis results in the formation of root nodules where infected nodule cells house differentiated bacteria termed bacteroids surrounded by a plant-derived symbiosome membrane (SM), forming a facultative organelle called the symbosome (1). The symbiosis results in the exchange of carbon (C) from the plant for NH₄⁺ produced through bacteroid nitrogenase activity (N₂ fixation). Legumes promote this relationship through the expression of specific symbiotic-enhanced nodule genes (2) that allow development of the symbiosis and importantly the exchange of nutrients between symbionts.

We previously identified Glycine max basic-helix–loop–helix membrane 1 (GmbHLHm1) (formerly GmSAT1) in a yeast complementation screen where it rescued growth of an NH₄⁺ transport mutant 26972c and enhanced NH₄⁺ transport was indirect, possibly associated with changes in abundance of the NH₄⁺ transport protein Mep3p. In this study, we used multiple approaches to demonstrate that GmbHLHm1 (SAT1), in contrast to results reported in Kaiser et al. (3) and in accordance with Marini et al. (4), does not encode an NH₄⁺ transporter but encodes a bHLH transcription factor (TF) that undergoes posttranslational modification for its delivery to the nucleus. In yeast, NH₄⁺ transport is activated through overexpression of MEP3 and a unique low-affinity NH₄⁺ transport protein common to both yeast and plants. The activity of GmbHLHm1 in soybean nodules indicates an important role in nodule development and growth that is linked ultimately to an effective N₂-fixing symbiosis.
acid motif (SI Appendix, Fig. S1) that recognizes the palindromic CANNTG promoter “E-box” element (6) commonly found in most bHLH-regulated genes. GmbHLHm1 homologs are found as multigene families in both dicot and monocot plant species (SI Appendix, Fig. S2).

TFs located outside the nucleus are rare (7, 8) and presumably positioned to respond to cellular signaling pathways (9). To verify its location, we used immunogold labeling with a polyclonal antibody (α-bHLHm1) (3). We identified GmbHLHm1 in various membranes [PM, endoplasmic reticulum (ER), Golgi, SM] (SI Appendix, Fig. S3A) and in the nucleus of infected nodule cells (Fig. L4). In GmbHLHm1-containing 26972c yeast cells, labeling was observed predominantly at the PM (Fig. 1B). Attachment of a N-terminal green fluorescent protein (GFP) tag to GmbHLHm1 in yeast resulted in GFP signal in peripheral punctate bodies but also in the nucleus (Fig. 1C–F), where nuclear localization was confirmed by cross-reacting with the vital DNA stain, Hoechst 33342 (Fig. 1D–F). Punctate and nuclear localization of GmbHLHm1 was also observed in plants when full-length GFP-GmbHLHm1 is transiently expressed in either Allium cepa (SI Appendix, Fig. S3B–D) or Nicotiana benthamiana epidermal leaf cells (SI Appendix, Fig. S3 E–H). Loss of the C-terminal TD (S294-V347) resulted in GFP signal only in the nucleus of N. benthamiana cells (SI Appendix, Fig. S3 J–M).

To understand the delivery of GmbHLHm1 to the nucleus, we used a modified split-ubiquitin assay involving the fusion of the artificial TF, LexA:VP16-Cub, to the N (LexA:VP16-Cub:GmbHLHm1) and C terminus (GmbHLHm1:LexA:VP16-Cub) of GmbHLHm1. When positioned at the N but not the C terminus, LexA:VP16-Cub complemented two nuclear reporter genes (HIS3 and lacZ) in the yeast strain DSY1 (SI Appendix, Fig. S3N). This indicated the N terminus of GmbHLHm1 is accessible to the cell cytosol, whereas the C terminus is not. To better understand the mechanism of release, we then introduced selected mutations (L277A and L277I) at a predicted site 1 proteolytic recognition site (RXXL: L277; SI Appendix, Fig. S1) positioned upstream of the C-terminal TD in both LexA: VP16-Cub:GmbHLHm1 and native GmbHLHm1. The mutations disrupted LexA:VP16 complementation of DSY1 and reduced MA toxicity in yeast strain 26972c (SI Appendix, Fig. S3 O and P). We interrogated total yeast protein from GmbHLHm1 or N-terminal GFP-tagged GmbHLHm1 cells with α-bHLHm1 and α-GFP antibodies, respectively. With both antibodies, we identified similar banding patterns (~37, 30, and 27 kDa: α-bHLHm1) (Fig. 1G, lanes 2 and ) (~66, 58, and 32 kDa: α-GFP) (Fig. 1H, lane 3). Based on the predicted size of GmbHLHm1 (3) without GFP, we believe the ~37-kDa band represents the native full-length protein, because we only identified the 30- and 27-kDa peptides in nuclear enriched fractions from native GmbHLHm1 yeast cells or when the C-terminal TD of GmbHLHm1 was deleted (Fig. 1G, lanes 3 and 5, respectively). The smaller 27-kDa protein is derived from a second uncharacterized proteolytic event at or after entry into the nucleus. Using A. cepa epidermal leaf cells, we profiled a full-length GFP-GmbHLHm1 construct against one where the N-terminal 21 aa of GmbHLHm1 was removed (~4 kDa). The N-terminal deletion altered GFP localization, where GFP::GmbHLHm1 was located in the nucleus, whereas full-length N-terminal GFP-tagged protein resided in the outer nuclear envelope (SI Appendix, Fig. S3 B–D).

GmbHLHm1 Is Important for Soybean Nodule Development. GmbHLHm1 expression is higher in nodules over nodule-detached roots (Fig. 2 A and B). When grown without rhizobium (minus N), GmbHLHm1 root expression is derepressed (Fig. 2B). In nodules, GmbHLHm1 expression increases with the onset of N₂ fixation (15 d after rhizobium inoculation) and then decreases as the nodules mature (Fig. 2A). We examined the diel expression of GmbHLHm1 across a 24-h period and found a nocturnal expression pattern (Fig. 2C). GmbHLHm1 is primarily expressed in the excrining nodule parenchyma, even though a fainter signal could be detected in the bacterial infected region of the nodule (Fig. 2D and E). Targeted RNAi silencing of the 3’-UTR of GmbHLHm1 (bhlhm1) using hairy root transformation reduced nodule numbers and nodule fresh weight (Fig. 2 H and I) (P < 0.05). In nodules that developed, N₂ fixation and/or N export was compromised, resulting in shoot chlorosis (Fig. 2 F–I). bhlhm1 nodules displayed reduced leghemoglobin (Fig. 2G) and a small-infection zone (Fig. 2 J–M), where infected cells were small with variable symbiosome development, whereas uninjected cells were often vacuolated (Fig. 2 L and M and SI Appendix, Fig. S4 F and G).

To profile translational changes in bhlhm1 nodules, we used quantitative (q) PCR and microarray analysis on isolated RNA from bhlhm1 and empty vector (vector) nodules (SI Appendix, Table S1 and Fig. S4 A–D). Although nodule development is impaired in bhlhm1 roots, we observed no change in expression of the bacteroid N₂-fixation genes NifH and FixU (10) (SI Appendix, Table S1 and Fig. S4 A–D).
and sat1-transformed nodules. IC, bacteroid-infected cell; UC, uninfected cell.

Fig. 2. Gene expression and loss of function of GmbHLHm1 in soybean nodules. (A) Developmental expression (DAP, days after planting) of GmbHLHm1 in N2-fixing soybean nodules and nodule-detached roots. (B) GmbHLHm1 expression in leaves, nodules, and nodule-detached roots with (R+) or without (R-) rhizobia from 32-d-old plants grown without nitrogen. (C) Diel expression of GmbHLHm1 in nodules. (D) and (E) Cellular expression identified using a GmbHLHm1 promoter::GUS fusion in both infected and uninfected nodule cells (D) and a nodulated root (E). IR, infected region; P, parenchymal cells; VT, vascular trace. (F) Chlorotic shoots of plants grown solely on sat1-nodulated roots supplied with nutrient solution containing no nitrogen. (G) Reduction in legume globulin in infected cells of sat1 nodules. (H) and (I) Reduction in nodule number (n = 19; P = 0.0125) and nodule fresh weight (n = 11; P = 0.0013) in bhlhm1 transgenic events. Data represent means ± SE. *P < 0.05. (U and K) Toluidine blue-stained fresh tissue cross-sections of vector and bHLHm1-transformed nodules highlighting small infected cells in bHLHm1 nodules. (L and M) TEM analysis of cross-sections of infected and uninfected cells from vector- and sat1-transformed nodules. IC, bacteroid-infected cell; UC, uninfected cell.
dimerization domains (L191V and L207V, two regions important for DNA binding (SI Appendix, Fig. S6)) disrupted [^{14}C]MA transport, MA toxicity, and expression of ScAMF1 (SI Appendix, Fig. S6E-G). Subsequent analysis of the ScAMF1 promoter identified eight predicted E-box binding domains (SI Appendix, Fig. S6F). Electromobility-shift analysis demonstrated that soluble GmbHLHm1 (128–270 aa) directly binds the ScAMF1 promoter (SI Appendix, Fig. S6F).

We examined the activity of ScAMF1 in both yeast and Xenopus laevis oocytes. In yeast, ScAMF1 increased [^{14}C]MA uptake and MA toxicity (0.1 M) in 26972c (Fig. 3 B and E). Many high-affinity NH$_4^+$ transport proteins, including Mep3p (4) and AMT1 (28), are capable of rescuing growth of 26972c on low (1 mM) NH$_4^+$ concentrations. ScAMF1 behaved differently; it failed to complement growth at 1 mM NH$_4^+$ (SI Appendix, Fig. S6C), suggesting an activity more suited to higher NH$_4^+$ concentrations. To test further, we injected ScAMF1 cRNA into X. laevis oocytes. ScAMF1 increased [^{14}C]MA uptake in a concentration-dependent manner (Fig. 3F) (P < 0.05; n = 10 oocytes). Using a two-electrode voltage clamp, increasing negative voltages across the oocyte PM resulted in a consistent NH$_4^+$ inducible inward current not present in the water-injected controls (Figs. 3 G and I).

Through sequence homology, we showed that AMF1 homologs are common in plants, including soybean, Medicago, Arabidopsis, and maize (SI Appendix, Figs. S7 and S8); interestingly, AMF1 homologs often display chromosomal microsynteny (29) with GmbHLHm1 genomic loci (SI Appendix, Fig. S9). We identified five AMF1 homologs in soybean consisting of two paralogous pairs: (i) Glyma15g06660 (GmAMF1) and Glyma13g32670 (GmAMF2); and (ii) Glyma08g06880 (GmAMF3) and Glyma07g30370 (GmAMF4); as well as Glyma09g33680 (GmAMF5). Analysis of publicly available RNA-seq data from Severin et al. (30) revealed GmAMF3 was up-regulated in nodules relative to roots (SI Appendix, Fig. S4E).

Over a 24-h diel cycle, GmAMF3 expression peaked mid-morning, whereas a second AMF, GmAMF5 expression was elevated in the afternoon and evening (SI Appendix, Figs. S5 G and H). We cloned a full-length GmAMF3 cDNA from 28-d-old soybean nodule mRNA and tested its functional activity relative to ScAMF1 in both yeast and Xenopus oocytes. In yeast, GmAMF3 increased [^{14}C]MA uptake relative to the empty vector control and ScAMF1 (Fig. 3H). In Xenopus oocytes, GmAMF3 elicited an NH$_4^+$ (5 mM)-dependent inward current when increasing negative voltages were applied across the oocyte PM (Fig. 3I). Based on the chemical and electrical signatures of ScAMF1 and GmAMF3, we suggest AMF proteins behave as NH$_4^+$ permeable transport proteins. We then examined the tissue localization of GmAMF3 in transformed soybean nodules and roots using the GmAMF3 (2.0-kB) promoter fused to the β-glucuronidase reporter GUS. GUS activity was primarily localized in cells surrounding the vascular bundles and the nodule parenchyma cell layer, which sits outside the infected zone (Fig. 3 K and L). We tested the intercellular location of GmAMF3 using a transient expression assay with YFP:GmAMF3 in onion epidermal cells. YFP: GmAMF3 was identified specifically on the PM similar to the PM localized AtPIP2a (31) (Fig. 3 M and N). In bhlhm1 nodules, we found no significant change in GmAMF3 expression; however, GmAMF5 (Glyma09g33680) was significantly (P < 0.05) down-regulated (SI Appendix, Fig. S4D).

Discussion

GmbHLHm1 is a Soybean Membrane-Localized DNA-Binding bHLH TF. One of the unique features of GmbHLHm1 is its membrane association when not in the nucleus. In soybean, GmbHLHm1 is located across multiple membranes in nodule infected cells, including the SM but also the PM, Golgi, and ER. Membrane association is also observed when the TF is heterologously expressed in other systems, including yeast, onion, and N. benthamiana leaf.
epidermal cells. Membrane bound TFs have been identified in plants, but less than 2% of the putative 1,533 TFs in Arabidopsis have been classified as membrane tethered (32). Examples of these include an Arabidopsis ER stress-responsive BZIP protein (33) and Arabidopsis nascent polypeptide-associated complex TFs (34). Membrane tethered TFs are assumed to be located outside the nucleus as a measure to regulate activity and to respond to cellular signaling pathways. Their activity is based on the anchored TF undergoing regulated intramembrane proteolysis or regulated ubiquitin/proteasome-dependent processing involving proteolytic cleavage and release of the soluble DNA-binding domain to the nucleus (9). Our data suggest that GmbHLHm1 can be delivered to the nucleus from its membrane anchor through a yet-unidentified proteolytic event upstream of its C-terminal TD (Fig. 1 H-J) and a predicted further modification at its N terminus, which may aid entry into the nucleus (SI Appendix, Fig. S3 C and D). This is consistent with other membrane-bound TFs, where release involves cleavage by membrane-associated serine, rhomboid, and possibly calpain-like proteases (9, 33–35). We observed via sequence comparison with other membrane bound TFs (OASIS, ATF2, SREBP2), a putative subtilisin site 1 proteolytic recognition site (RXXL: L277) upstream of the C-terminal TD of GmbHLHm1 (SI Appendix, Fig. S1). Substitutions at this site (L277A, L277I) reduced MA sensitivity and prevented nuclear entry of the artificial TF LexA-VP16 (SI Appendix, Fig. S3 O and P). Transcriptional activity of GmbHLHm1 increased the expression of a select number of yeast genes involved in nutrient transport, phosphorus homeostasis and cell wall modification (SI Appendix, Table S2). In particular, we identified an uncharacterized yeast gene ScAMF1 (YOR378w) that was strongly up-regulated by GmbHLHm1 (SI Appendix, Table S2). We confirmed this regulation was dependent on the DNA-binding domain of GmbHLHm1 through site-directed mutagenesis and through electromobility-shift analysis that indicated an affinity of purified GmbHLHm1 protein to the promoter of ScAMF1 (SI Appendix, Fig. S6f).

GmbHLHm1 Activity Identifies a Unique Low-Affinity NH₄⁺ Transport Protein in Yeast and Plants. Discrepancies between previous interpretations of GmbHLHm1 (SAT1) acting as an independent NH₄⁺ transport protein (3), or not (4), were resolved in this study by demonstrating that GmbHLHm1 (SAT1) is instead a bHLH TF that enhances the expression of the high-affinity NH₄⁺ transporter, MEP3 (SI Appendix, Fig. S6a), a phenotype that explains the ability of GmbHLHm1 (SAT1) to complement growth of yeast mutants 26972c but not 31019b on low NH₄⁺ concentrations. Because MEP3 does not present E-box binding domains (6) in its promoter region, the enhanced expression by GmbHLHm1 may be indirect. This could be mediated by altered cellular NH₄⁺ homeostasis influenced by the GmbHLHm1-enhanced expression of the NH₄⁺ channel ScAMF1, which our data indicate is responsible for both MA uptake and MA toxicity when GmbHLHm1 is expressed in either 26972c (3) or 31019b (Fig. 3 B–E).

The identification of ScAMF1 reveals a previously unknown mechanism by which yeast cells can manage NH₄⁺ transport. We have shown ScAMF1 is a yeast PM protein that is capable of facilitating MA uptake (Fig. 3 B and E). We have verified its transport activity further in X. laevis oocytes using both chemical (MA uptake) and electrical (NH₄⁺-induced ion currents) studies. Incubating oocytes with increasing concentrations of [¹⁴C]MA significantly enhanced MA uptake across both high-affinity (<250 µM) and low-affinity (>250 µM) ranges. Similarly, we observed inward electrical currents at high concentrations (5 mM) of NH₄⁺ in ScAMF1 CRNA-injected oocytes (Fig. 3G). Collectively, these studies in both yeast and Xenopus suggest ScAMF1 is most likely a low-affinity NH₄⁺ transport protein. Its role in yeast NH₄⁺ transport/metabolism is still to be defined. Mep(1-3)p transporters are clearly involved in the high-affinity uptake of NH₄⁺ and are required for growth at low NH₄⁺ concentrations (23). However, the mechanisms responsible for low-affinity NH₄⁺ transport (physiological and genetic) have until now been poorly understood. Our data suggest ScAMF1 is most likely a participant in this process.

We identified AMF1 homologs across multiple plant genomes (SI Appendix, Fig. S8). Soybean contains five, where four are physically associated (within 20 kb) with GmbHLHm1 loci. Publicly available RNA-seq expression data in soybean (30) demonstrated that GmAMF3 (Glyma08g06880) is nodule enhanced. GmAMF3 is primarily expressed in nodule parenchyma cells and the enveloping vascular tissues. Similar to ScAMF1, functional expression of GmAMF3 in both yeast and X. laevis oocytes resulted in [¹³C]MA and NH₄⁺ transport activity (Fig. 3 H and I). This result suggests that a low-affinity NH₄⁺ channel may operate in both the nodule parenchyma and vascular cells that connect the N₂-fixing inner cortical cells to the root. Because the majority of fixed nitrogen (N) exported from soybean nodules are ureides (36), the requirement of an NH₄⁺ channel in these cell types is unclear. Previous studies in Lotus japonicus have shown that PM localized high-affinity (AMT) NH₄⁺ transporters are expressed across nodule-injected, vascular, and outer parenchyma cells (37, 38). Together, this would suggest NH₄⁺ is present in many cell types of the nodule and that transport systems are required for efficient NH₄⁺ recapture and assimilation or potentially release from the nodule through excessive N₂ fixation (39).

GmbHLHm1 Activity is Important to the Rhizobium/Legume Symbiosis. GmbHLHm1 expression occurs primarily in the nodule parenchyma cells, as GmAMF3, but is also weakly detected in the infected region of the nodule and shows differential expression between roots exposed to or grown in the absence of rhizobia. GmbHLHm1 expression in nodules occurs during the night and decreases during the day. At the intercellular level, GmbHLHm1 is associated with the SM but also the PM, Golgi, ER, and the nucleus, which introduces an interesting link between membrane-based signaling and transcriptional activity in the nucleus. Apart from the association with AMF1, GmbHLHm1’s activity in legume nodules suggests an important role in the success of the symbiosis. Loss of bhlm1 in soybean roots disrupted nodule development, resulting in reduced nodule numbers, which were generally smaller with impaired symbiosome development. These developmental changes were accompanied by a modest shift in transcriptional expression in the nodule that included down-regulated genes involved in the maintenance of auxin concentrations through amino acid conjugation (GH3/BRU6) and cell wall xyloglucan modification (XTH) and, interestingly, the regulation of the morning and evening components of the circadian clock (GT1, PRR5, and PRR7) activity suggested an important role in the success of the symbiosis. Loss of GmbHLHm1 in soybean roots disrupted nodule development, resulting in reduced nodule numbers, which were generally smaller with impaired symbiosome development. These developmental changes were accompanied by a modest shift in transcriptional expression in the nodule that included down-regulated genes involved in the maintenance of auxin concentrations through amino acid conjugation (GH3/BRU6) and cell wall xyloglucan modification (XTH).

The role of the circadian clock in N₂-fixing legume nodules is unknown, but we assume it is similar to that of roots and linked to an underlying metabolic control regulating growth (15, 40). The circadian clock is also linked to the global regulation of endogenous auxin signaling involving auxin-induced genes in Arabidopsis, including homologs of the auxin-amido synthetases (41) found down-regulated in bhlm1 nodules. How this relates to the role of BHLHm-like TFs identified in nonlegumes is less clear (SI Appendix, Fig. S3). In Arabidopsis, a BHLHm1 homolog, At2g22770 (NAI1), is involved in stress-induced synthesis of ER bodies in root and leaf tissues (42). It is possible that BHLHm1 TFs may have a role in facilitating other microbial interactions, including pathogen responses or mycorrhizal symbioses, both of which involve C supply from the plant. The identification of GmbHLHm1 presents an exciting opportunity to begin unraveling the subtle interactions that circadian rhythms may play in the establishment and maintenance of the rhizobium/legume symbiotic partnership.

In conclusion, we propose that GmbHLHm1 is an important plant regulator used for the rhizobium/legume symbiosis. Activity in yeast is linked to NH₄⁺ transport through AMF1 activity, whereas AMF1 and GmbHLHm1 share chromosomal conservation across many plant species, including soybean. The link with nodule-expressed circadian clock genes suggests a role of GmbHLHm1 in mediating the underlying symbiosis-specific exchange of C from the plant for fixed N from rhizobia. Because bhlm1 sequences are
present in plants, but not in lower-order eukaryotes or prokaryotes, we suggest that bHLHm1-like proteins are likely an adaptation involved in mediating plant-microbe interactions.

Materials and Methods

Plant Materials and Growth Conditions. Soybeans (Glycine max L. cv. Djakal) were grown in sand in either a growth chamber (28/25 °C, 16-8-h daytime/night regime) under mercury halide lights (∼600 photosynthetic active radiation) or a temperature-controlled glasshouse (25/15 °C, day/night temperature) supplemented with mercury halide lights (16-8-h daytime/night regime). Plants were inoculated with Bradyrhizobium japonicum US Department of Agriculture (USDA) 110 and watered with N-free nutrient solution. See SI Appendix, SI Materials and Methods for details.

Cellular Localization and Expression of GmbHLHm1. The cellular localization of GmbHLHm1 was defined using immunogold labeling with anti-GmbHLHm1 polyclonal antibodies in wild-type nodules and yeast. Promoter-GUS fusion constructs were expressed in nodulated hairy roots generated with Agrobacterium rhizogenes. In planta GFP tagging experiments were carried out in N. benthamiana using particle bombardment of N. benthamiana in polyclonal antibodies in wild-type nodules and yeast. Promoter-GUS fusion were inoculated with yeast supernatants and cultivated under 16/8-h day/night regime. Plants were inoculated with Bradyrhizobium japonicum US Department of Agriculture (USDA) 110 and watered with N-free nutrient solution. See SI Appendix, SI Materials and Methods for details.

Modification of GmbHLHm1 Activity in Soybean Nodules and Yeast Cells. GmbHLHm1 expression in soybean roots and nodules was repressed using a 3′-UTR hairpin loop inserted into the soybean genome via A. rhizogenes root transformation (43). Modification of the putative bHLH DNA-binding and dimerization domain of GmbHLHm1 was evaluated using site-directed mutagenesis. AAM1-mediated transport activities were measured using yeast NH₄⁺ transport mutants (26972c, 31019b) and cRNA AMF1-injected I. laevis oocytes with [³¹]Ca influx analysis and/or two-voltage electrode clamping. See SI Appendix, SI Materials and Methods for details.

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