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The gibberellic acid responsive GmbHLHm1 transcription factor influences nodule development, nitrogen fixation activity and shoot nitrogen content in soybean (*Glycine max*)

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ABSTRACT

GmbHLHm1 is a basic Helix-Loop-Helix membrane (bHLHm1) DNA binding transcription factor localized to the symbiosome membrane and nucleus in soybean (*Glycine max*) nodules. Overexpression of *GmbHLHm1* significantly increased nodule number and size, nitrogen fixation activity, and nitrogen delivery to the shoots. This contrasts with reduced nodule numbers per plant, nitrogen fixation activity and poor plant growth when silenced using RNAi. The promoter of *GmbHLHm1* was found to be sensitive to exogenous GA supply, decreasing the level of GUS expression in transformed hairy roots in both nodules and roots and reducing native *GmbHLHm1* expression in wild-type nodules. In summary, our study suggests that *GmbHLHm1* positively regulates soybean nodulation and nitrogen fixation, and that GA can negatively regulate *GmbHLHm1* expression in soybean nodules.

Keywords: ammonium, gibberellic acid, *Glycine max*, GmbHLHm1, nitrogen, nitrogen fixation, nodulation, soybean, symbiosome.

Introduction

Soybean (*Glycine max*) is an important agricultural crop used in the production of plant-based oils and proteins, animal feeds, food ingredients, and biofuels (Anderson *et al.* 2019). Soybean is a nitrogen-fixing legume capable of forming a symbiosis with soil-borne rhizobacteria. Compatible rhizobia infect root epidermal cells initiating the formation of specialised organs called root nodules which house the N_2-fixing rhizobia called bacteroids (Ferguson *et al.* 2019; Li *et al.* 2020). Bacteroids receive carbohydrates from the plant to energise the fixation of atmospheric N_2 to NH_3 by the bacteroid enzyme, nitrogenase. NH_3 is readily assimilated by the plant providing a sustainable Nitrogen (N) resource that can supplement or even replace the need for N fertilisers used in agricultural production systems (Kebede 2021; Barbieri *et al.* 2023).

GmbHLHm1 (*Glyma. 15g061400*) encodes a DNA-binding transcription factor (TF) that is expressed in soybean root nodules and non-nodulated root cells (Chiasson *et al.* 2014). In nodules, its expression is enhanced upon the initiation of symbiotic N_2-fixation (Chiasson *et al.* 2014) with GmbHLHm1 protein found localised on membranes, including the symbiosome, Golgi and plasma. As a TF, GmbHLHm1 is also found localised to the nucleus of infected nodule cells (Kaiser *et al.* 1998; Chiasson *et al.* 2014). In roots, *GmbHLHm1* is predominantly located in the vascular cells increasing in expression when starved of N. Loss of *GmbHLHm1* using RNAi compromises nodule development and symbiotic N_2-fixation (Chiasson *et al.* 2014). Recently, we have identified a role of a *GmbHLHm1* orthologue in the symbiotic arbuscular mycorrhizal symbiosis in *Medicago truncatula* (Ovchinnikova *et al.* 2023). Loss of *MtbHLHm1* expression reduces the mycorrhizal growth response and the mycorrhizal colonisation of infected roots. MtbHLHm1 was found localised to arbuscule containing cells and located across the plasma membrane and nucleus of arbuscule penetrated cells (Ovchinnikova *et al.* 2023). The MtbHLHm1 TF was also able to bind to the promoter of the ammonium facilitator protein *MtAMF1;3* which is also expressed in arbuscule containing infected cells (Ovchinnikova *et al.* 2023).

Gibberellins (GAs) are plant hormones that regulate cell and tissue growth and influence various developmental processes including plant height (Sasaki *et al.* 2002) and nodulation (Velandia *et al.* 2022). Bioactive GAs are recognised by the GID1 (GA INSENSITIVE DWARF1) receptor protein (Ueguchi-Tanaka *et al.* 2005; Hartweck and Olszewski 2006). GA-activated GID1 proteins bind DELLA proteins, which are transcriptional regulators of GA-induced responses in plants (Murase *et al.* 2008; Harberd *et al.* 2009). Studies have reported that the transcripts of gibberellic acid 20-oxidase (GA20ox), which is crucial for bioactive GA synthesis, are upregulated when nodulation occurs on adventitious and lateral roots in a Nod factor-dependent manner in *Sesbania rostrata* (Lievens *et al.* 2005). Several GA biosynthesis genes, *GmGA20ox1a, GmGA3ox1a,* and *GmGA2ox1a* are also reported to be upregulated during the early nodulation stage in soybean roots (Hayashi *et al.* 2012; Chu *et al.* 2022). Unsurprisingly, many rhizobia species such as *Bradyrhizobium japonicum* produce GA to promote nodulation (Boiero *et al.* 2007; Nett *et al.* 2022).

GA-responsive elements, pyrimidine box (P-BOX) and TATC-BOX (TGGGATA), and auxin-responsive TGC elements (AACGAC) have been identified in the promoter region of *GmbHLHm1* (Mohammadi Dehcheshmeh 2013). P-BOX motifs were first characterised as a regulator of GA-responsive genes in the barley aleurone (Mena *et al.* 2002), while sequence analysis of the *Brassica napus* GA-insensitive dwarf mutant *ndf1*, identified a mutation in the P-BOX motif of the promoter region of the GA receptor *BnGID1* (Li *et al.* 2011). Similarly, A TATC-BOX (TGGGATA) in the promoter of 4-Coumarate-CoA ligase (4CL) in *Pennisetum purpureum* has been demonstrated to interact with gibberellic acid (GA). This interaction was confirmed through a combination of deletion analysis, electrophoretic mobility shift assay (EMSA) binding studies involving GA, and experiments using promoter fusions with the β-glucuronidase (GUS) reporter gene (Peng *et al.* 2016). The GA-responsive elements identified in the *GmbHLHm1* promoter suggests a possible regulatory control mechanism that connects endogenous GA to the regulation (positive or negative) of *GmbHLHm1*.

In this study, we examined the role of GmbHLHm1 in the development and activity of soybean nodules utilising both reverse and forward genetics. Loss of expression compromises nodule growth and function, while overexpression of *GmbHLHm1* leads to enhanced nodule growth per plant, nitrogen fixation rates and the % shoot nitrogen. The application of exogenous GA negatively impacts *GmbHLHm1* expression levels in soybean nodules and roots.

Materials and methods

Seeds of soybean (*Glycine max* L. Merr.) cv. Snowy were sourced from the NSW Department of Primary Industries and surface sterilised by soaking in 1:2 (v/v) water diluted bleach for 2 min and rinsed 3 times with autoclaved water. Surface-sterilised seeds were then transferred to plates with autoclaved Turface (Turface Athletics, USA). Plates were covered with cling wrap to prevent contamination and to maintain humidity and then placed in an incubator with a 14/10 h day/night regime with light intensity at 200 μmol m^{-2} s^{-1}) photosynthetic flux density at plate level. Seeds were germinated in a day/night temperature cycle of 28–25°C for 7–10 days. Seedlings that had germinated to a length of 3–5 cm were chosen for hairy root transformation. This process was carried out using *Agrobacterium rhizogenes* strain K599, following the protocol established by Mohammadi-Dehcheshmeh *et al.* (2014). All hairy roots were inspected for GFP fluorescence, and any non-transformed roots were excised. Plants with transgenic hairy roots were transferred to individual pots (1 L) with a mixed matrix of quartz sand and Turface at 1:1 ratio and grown within covered plastic lids for 2 days at 28°C under light intensity of 400 PAR. After 2 days, the plants were placed into the chamber and inoculated. For nodulation, 50 mL of NoduleN (New Edge Microbiol, Australia) legume inoculant strain CB 1809 (Botha *et al.* 2004) was mixed in 1 L of distilled water, and 20 mL of the inoculant solution applied to each seedling with a syringe. A nitrogen-free (–N) B&D nutrient solution (Broughton and Dilworth 1971) was applied twice a day using an automated semi-hydroponic system. Where required, 5 mM KNO_3 was added to the –N B&D nutrient solution. Plants were grown in a controlled growth chamber with 14/10 h day/night regime, 25/22°C day/night temperature cycle, and 60% humidity for a minimum of 28 days. Seedlings with 28-day-old nodules (days after inoculation) were harvested for further experiments. Root, shoot or nodule samples were also dried at 60°C for 4 days before dry weights (DW) were measured.

Gene silencing and overexpression of *GmbHLHm1*

For RNAi-mediated gene silencing, a 359-bp porttion of the *GmbHLHm1* 3′UTR (*Glyma. 15g061400*) was amplified and inserted into the pK7GWIWG2D(II) vector (Karimi *et al.* 2002; Chiasson *et al.* 2014). For overexpression, the full-length *GmbHLHm1* CDs (1048 bp) was amplified from reverse transcribed soybean nodule total RNA with primers (forward: GTCCGCGGATGAGGAGTTCTCATATGGAGA) and (reverse: TGGCGCGCCTCACACGAAATATGAAAAAGCT) primers that incorporated a *Sac* II restriction site on the 5′ end and an *Asc* I restriction site on 3′ end. The full CDs was inserted into pENTR by double digestion (*Sac* II and *Asc* I, NEB) followed by incorporation using T4 ligation (Thermofisher Scientific™). The full-length *GmbHLHm1* CDs was then inserted into the pFAST-G02 (Shimada *et al.* 2010) with the Gateway system and introduced into soybean roots by the *A. rhizogenes*-mediated hairy root transformation as described by Mohammadi-Dehcheshmeh *et al.* (2014).

GA treatment of wild-type and nodulating and non-nodulating hairy roots

GA_3 was applied to wild-type soybeans or seedlings with transgenic hairy roots 5 days after inoculation with *Bradyrhizobium japonicum* (CB 1809). GA was applied as 4 ppm (10^{-5}M) GA_3 directly to the soil twice a week (Sudadi and Suryono 2015), a similar volume of water was added to the control groups. The surface of the pots were covered with aluminium foil over the treatment period and the plants harvested and analysed at 28 days. *GmbHLHm1Pro:GUS* transformed plants were supplied GA_3 (4 ppm) at 23 days after inoculation with rhizobia, and harvested and analysed at 28 days.

RNA extraction and quantitative PCR (qPCR) analysis

Plants were harvested at 11:00 hours. Nodules were detached from the roots and both tissues immediately frozen in liquid N_2 before being transferred to a −80°C freezer. RNA extraction was performed using the PureZOL total RNA extraction reagent (Bio-Rad). First-strand cDNA synthesis was performed using iScript cDNA Synthesis Kits (Bio-Rad). Primers for *GmbHLHm1* expression (forward: GCTCGGTGATAACAGCT GGA; reverse: CACGCCATCTCCACCTTAGG) were designed using Geneious software (Geneious). Primer efficiency was tested with SYBR Green Real-Time PCR Master Mix and 1, 1/5, 1/25, 1/125, 1/625 dilution of cDNA synthesised. The primer efficiency was 90–100%. 2 μL of a 1/5 dilution of cDNA was used as the qPCR templates. SYBR Green Real-Time PCR Master Mix was used for all qPCR experiments. Results were normalised against *Cons6* as the reference gene and calculated using the $2^{-\Delta\Delta Ct}$ method (Libault *et al.* 2008).

Measurement of nodule N_2-fixation and %N in plant tissues

Intact root systems (transgenic roots and nodules) were collected in the morning (~11:00 hours) and placed individually in 40-mL McCartney vials sealed with a rubber septum. A 5-mL gas seal syringe was used to draw out 4 mL of air from the sealed vial, and 4 mL of instrument grade, dissolved acetylene gas (BOC, Australia) was injected into the vial with another gas-sealed syringe. The starting acetylene-ethylene levels in the vial were measured with GC-2010 Plus gas chromatograph (SHIMADZU, Japan). Vials were then incubated in a 28°C water bath for 1 h and 1 mL extracted and measured for acetylene and ethylene levels with a GC-2010 Plus gas chromatograph (Shimadzu) against a ethylene standard curve prepared from pure (99.99%) Ethylene (Sigma-Aldrich). The samples were removed from the vials, nodules were detached from the roots and placed in a 60°C oven to dry overnight. Dry nodules were weighed, and the nodule dry weight (DW) data were used to calculate the rate of acetylene reduction over time following the methods of Unkovich *et al.* (2008). To measure the %N in harvested tissues, the top five leaves were sampled and dried at 60°C for 4 days and then ground to a fine powder. A 2-mg sample of powdered leaves was transferred to a tin capsule and the %N content in the plant samples determined using an isotope ratio mass spectrometer (Sercon, Crewe, Cheshire, UK) as described previously by Dechorgnat *et al.* (2018).

Promoter-GUS fusion construction and GA motif editing

Genomic DNA of soybean cv. Snowy was isolated from nodules using the PureLink Genomic Plant DNA Purification Kit (Thermo Fisher). A 1926-bp section upstream of the *GmbHLHm1* (Glyma15g06680) start codon was cloned using primers (forward: AGCATGGCCGTGATTTAACCTAAG AAAACCAATTC; reverse: GACGTAACATTATACTCAAACTA CAACATCC). The promoter was cloned into the pCR8 vector and then recombination cloned into the pKGWFS7 vector with the Gateway cloning system (Karimi *et al.* 2002). The *P-Box1*, *TATA-Box* and *P-Box2* motifs were edited separately into polyadenylate (aaaaaaa) repeat sequences using PCR primers described in Table 1.

Constructs were transformed into the *A. rhizogenes* K599 strain. The Promoter-GUS fusion construct was used for hairy root transformation and cultivation with the method of Mohammadi-Dehcheshmeh *et al.* (2014). GUS staining was performed on transformed roots according to (Chiasson *et al.* 2014) with modifications. The harvested transgenic hairy roots with nodules were soaked in 90% (v/v) ice-cold acetone in a 50-mL centrifuge tube. Each sample was rinsed twice in sodium phosphate buffer for 5 min, before being transferred into GUS staining buffer. GUS staining buffer-covered samples were transferred in a vacuum for infiltration for 30 min and then incubated at 37°C for 5 h.

Table 1. PCR mutagenic primers to alter GA-binding motifs in the *GmbHLHm1* promoter.

Primer ID	Primer (5′–3′)
p-box1f	aaaaaaaGTAAAAATGAGTTGGGCAAATAACCTTTG
p-box1r	TTCATTATTTTCTAAGTTCCTTTCTTAGATCCT
tatc-boxf	aaaaaaaAGTCTATTATCCTTGGTTGAAAATAGGC
tatc-boxr	TTTTATATTTTAGAGACCCCTTCATGCTG
p-box2f	aaaaaaaAATTACAACAACGAAATATATAATCATCAGCTTC
p-box2r	TATACTCTACCCCACTGTATTACAGCATATAAC

Results

RNAi-mediated gene silencing of *Gmbhlhm1* inhibits nodulation and N_2-fixation

To confirm the function of GmbHLHM1 in root nodulation and N_2-fixation, we re-employed RNAi to silence *Gmbhlhm1*. This post-transcriptional gene silencing (PTGS) approach utilised the binary vector pK7GWIWG2D(II), which contained a *Gmbhlhm1* silencing cassette under the control of the constitutive 35S promoter. The construct was introduced into soybean plants through *A. rhizogenes*-mediated hairy root transformation, allowing for targeted gene silencing in the root system. We verified a reduced expression level in the RNAi line (*Gmbhlhm1*) of approximately 50% of either the empty vector control and wild-type plants through qPCR on extracted total RNA from harvested nodules (Fig. 1*a*). Our previous report (Chiasson *et al.* 2014) characterised the function of *GmbHLHm1* in the soybean cv. Djakal. In this study, we observed a consistent phenotype when silencing of *GmbHLHm1* in a different soybean cultivar (Snowy), confirming this function of *GmbHLHm1* in the rhizobia symbiosis is not cultivar dependent. With reduced *GmbHLHm1* expression, total plant nodule weight, nodule number, and the ratio of nodule number to root DW were reduced significantly compared to the controls (Fig. 1*b–d*). There was no significant change in average nodule size in the *Gmbhlhm1* plants (Fig. 1*e*). After 28 days of growth, most of the initial soybean nodules have begun to fix N_2 (Herridge *et al.* 1990; Imsande 1991; Bergersen *et al.* 1992). Qualitative images of representative *Gmbhlhm1*-silenced lines showed yellowed leaves and a root system with fewer nodules (Fig. 1*h–j*), while empty vector and wild-type plants appeared healthy. The *Gmbhlhm1*-silenced nodulated hairy roots resulted in reduced shoot height, shoot DW and root DW at 28 DAI (Fig. 1*k–m*). Each of these growth phenotypes could be restored when grown uninoculated with applied N (5 mM KNO_3) (Fig. 1*k–m*). The silenced nodules showed a reduced effective N_2-fixation area (pink area; Fig. 1*j*) compared to wild-type and empty vector controls. Pink colour in legume root nodules is indicative of the presence of leghaemoglobin, a crucial protein that plays a vital role in N_2-fixation in legume root nodules (Ott *et al.* 2005). Altered nodule development resulted in significant reductions in N_2-fixation (measured via the acetylene reduction assay) (4-fold) and a decline in the %N present in harvested leaves (measured by mass spectrometry) (four fold) relative to the empty vector controls (Fig. 1*f*, *g*).

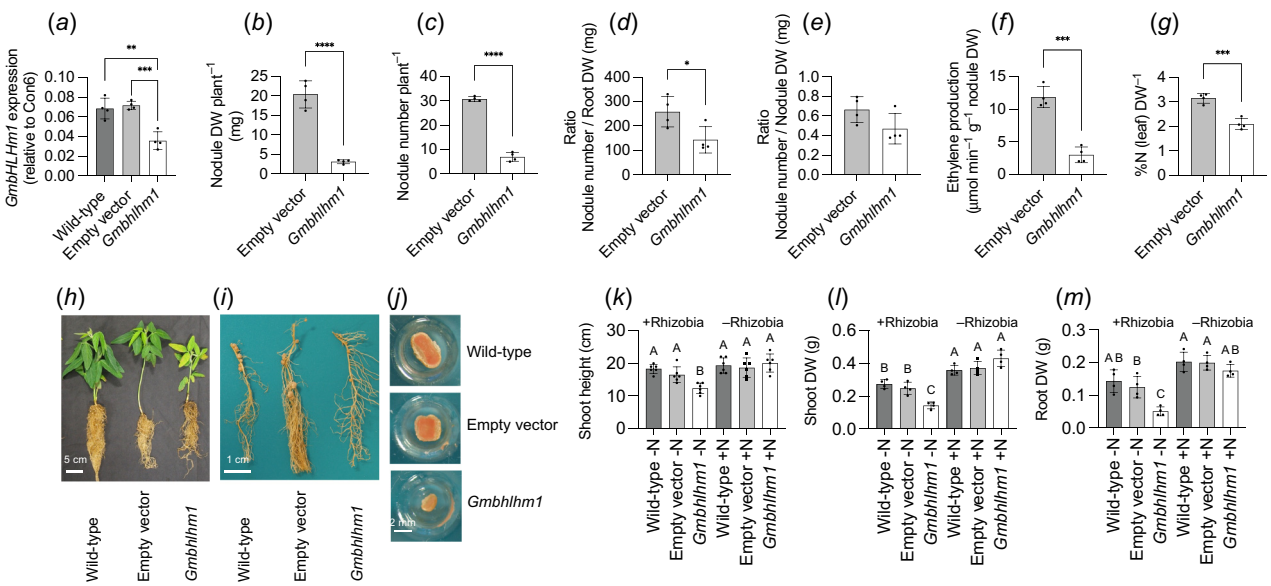

Fig. 1. Impact of *Agrobacterium rhizogenes*-mediated post-transcriptional gene silencing of *Gmbhlhm1* on composite plants of soybean with and without inoculation with rhizobia. (*a*) *GmbHLHm1* expression in wild-type, empty vector and *Gmbhlhm1* RNAi-silenced nodules. Reduction in expression of *Gmbhlhm1* in nodulated hairy roots reduced (*b*) nodule DW per plant, (*c*) nodule number per plant, (*d*) ratio of nodule number to root DW per plant, (*e*) ratio of nodule number to nodule DW, (*f*) ethylene production from nodulated roots measured using the acetylene reduction assay, and (*g*) %Nitrogen (N) in leaf DW. Qualitative and representative images of 28 DAI (days after inoculation with *Bradyrhizobium japonicum* (CB 1809) in (*h*) wild-type, Empty Vector, and *Gmbhlhm1 RNAi* whole plants. (*i*) Nodulated roots and (*j*) nodule cross sections. (*k*) Shoot height, (*l*) shoot DW, and (*m*) root DW ± rhizobia of inoculated wild-type, empty vector, and *Gmbhlhm1* plants supplied without (–N) or with (+N) 5 mM KNO_3 for 28 days. The expression of *GmbHLHm1* was normalised with *Con6* as a reference gene and was calculated using the $2^{-\Delta\Delta Ct}$ method (Libault *et al.* 2008). Values were means ± s.d. (n = 4–5 individual plants). Significance was determined using either a one-way ANOVA with Sidak *post hoc* test for multiple comparisons (*a*, *k*, *l*, *m*) or an unpaired *t* test (*b–g*). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

GmbHLHm1 expression responds to GA treatments

Three putative GA responsive elements (*P-Box1*, *TATA-Box*, *P-Box2*) were identified in the promoter region of *GmbHLHm1* (Fig. 2*a*) using the Plant-CARE database (Lescot *et al.* 2002). To test their potential role in GA regulation of *GmbHLHm1*, a full-length *GmbHLHm1* promoter (1926 bp upstream of the start codon) was cloned from genomic DNA and inserted upstream of the reporter GUS in the pKGWFS7 (Promoter-GUS reporter) vector to generate transgenic hairy roots and nodules after inoculated with *B. japonicum* (CB 1809). After 23 days, nodulated roots were treated daily with 4 ppm GA_3 or with water (control) for 5 days. At 28 days, nodules attached to roots were harvested to measure GUS expression. As shown in Fig. 2*a*, the GA treatment reduced the level of GUS staining in both nodules (Fig. 2*a*, *e*) and roots (Fig. 2*a*), compared to the water treated controls. Quantification of gene expression (qPCR) revealed that GA significantly reduced transcript levels of the *GmbHLHm1* promoter-GUS transgene in both roots ($P < 0.0017$) and nodules ($P < 0.0085$) (Fig. 2*b*, *c*). Application of GA_3 to empty vector controls, reduced endogenous *GmbHLHm1* expression after 1 ($P < 0.0014$) and 24 h ($P < 0.0001$) exposure (D). Editing of any of the three proposed GA elements in the *GmbHLHm1* promoter completely disrupted promoter activity, eliminating GUS expression in hairy-root derived nodules (Fig. 2*e*). Collectively, the qPCR and GUS staining experiments indicates a negative relationship between long-term GA exposure and *GmbHLHm1* promoter activity (reduced GUS expression) and gene expression. Promoter editing indicates that all three GA-motifs are required for promoter functionality but does not show a direct relationship between GA and the proposed *GmbHLHm1* GA promoter elements.

Overexpression of *GmbHLHm1* increased nodule size and nodule N_2-fixation

GmbHLHm1 was overexpressed (*GmbHLHm1 OEX*) in hairy roots ($P < 0.05$ relative to the empty vector control) using a

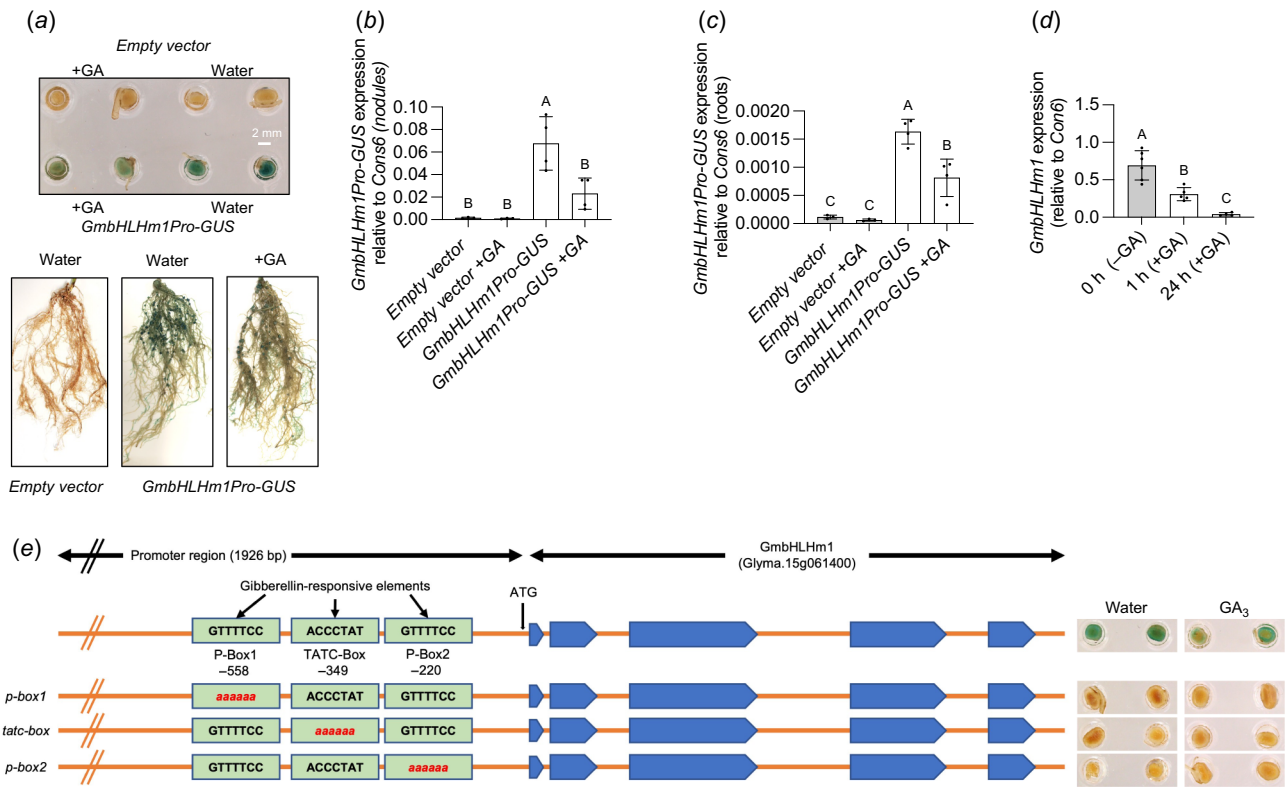

Fig. 2. GA recognised elements in the promoter of *GmbHLHm1* (Glyma. 15g061400). (*a*) GA repression of *GmbHLHm1Pro-GUS* in GUS stained *GmbHLHm1Pro-GUS* hairy root nodules and roots with the application of GA (4 ppm GA_3) or water twice per week. Corresponding *GUS* expression in *GmbHLHm1Pro-GUS* and empty vector control (*b*) nodule and (*c*) root tissues. (*d*) Changes in empty vector control *GmbHlHm1* expression (18 days nodules) in response to short-term (1 and 24 h) of 4 ppm GA_3 treatment. (*e*) Diagram highlighting three GA-responsive DNA elements (P-Box1, TATC-Box, and P-Box2) in the upstream region of the *GmbHLHm1* promoter. Loss of GA element function in nodules through selected nucleotide substitutions across *p-box1*, *tatc-box* and *p-box2*. The expression of *GUS* was normalised with *Con6* as a reference gene and was calculated using the $2^{-\Delta\Delta Ct}$ method (Libault *et al.* 2008). Values were means ± s.d. (n = 3–6 individual plant events). Significance was determined using one-way ANOVA (*b*, *c*, *e*) with a multiple comparison test (Sidak). Different letters above bars indicate a level of significance ($P < 0.05$).

35S promoter (Fig. 3*a*). At 28 days after rhizobia inoculation, changes in nodule development were evident, including significant increases ($P < 0.05$) in total nodule DW per plant (Fig. 3*c*), nodule size (Fig. 3*d, j*), N_2 -fixation rates (ARA activity) (Fig. 3*e*), %N in aerial leaf tissues (Fig. 3*f*). and shoot DW (Fig. 3*g*). There were no changes in either nodule number per root system, root DW or shoot height in *GmbHLHm1* OEX plants (Fig. 3*b, h, i*, respectively).

Discussion

Legume root systems have two roles in plant N acquisition strategies: (1) the uptake and assimilation on exogenous N (inorganic and organic) from the soil solution; and (2) the establishment of a symbiotic N_2-fixation symbiosis with soil-borne rhizobia bacteria. Both activities result in the transport of reduced N (NH_4^+, NO_3^-, and amino acids) to developing shoot and root tissues. *GmbHLHm1* expression is linked to nodule development and activity (Chiasson *et al.* 2014). For plants grown without N or when grown uninoculated, *GmbHLHm1* expression was shown to be strongly upregulated, while repressed when inoculated with N_2-fixing rhizobia (Chiasson *et al.* 2014). We employed a post-transcriptional gene silencing (PTGS) approach to reduce the expression of *GmbHLHm1* in soybean roots. As first observed by Chiasson *et al.* (2014), the reduction of *GmbHLHm1* expression in hairy roots led to poorly developed nodules, and poor growth of the plant when grown in the absence of N fertilisers. Our study further detailed this response, showing a reduction in nodule DW, nodule number per plant and a significant decrease in the rate of nitrogenase activity and a corresponding reduction in the %N content in the shoots (Fig. 1). As a result, *Gmbhlhm1*-RNAi plants grown solely on nodule-derived N showed symptoms of N deficiency such as a yellowing of the leaves and reduced shoot growth (Fig. 1*h*) relative to the empty vector controls and wild-type plants. Interestingly, we show for the first time that shoot height, shoot and root growth (DW) in the *Gmbhlhm1*-RNAi lines can be recovered with the supply of exogenous nitrogen fertiliser (5 mM KNO_3^-), which suggests the growth deficiencies are linked to *Gmbhlhm1*-RNAi compromised nodule activities and not changes in root N assimilation or root N redistribution (Fig. 1*k–m*).

When *GmbHLHm1* was overexpressed with the constitutive *35S* promoter in a hairy-root transformation system, nodule weight (DW) and nodule size increased relative to the empty vector controls (Fig. 3*c, d*) with larger older nodules evident on the root system (Fig. 3*j*). This translated into higher rates of N_2-fixation, the %N observed in aerial

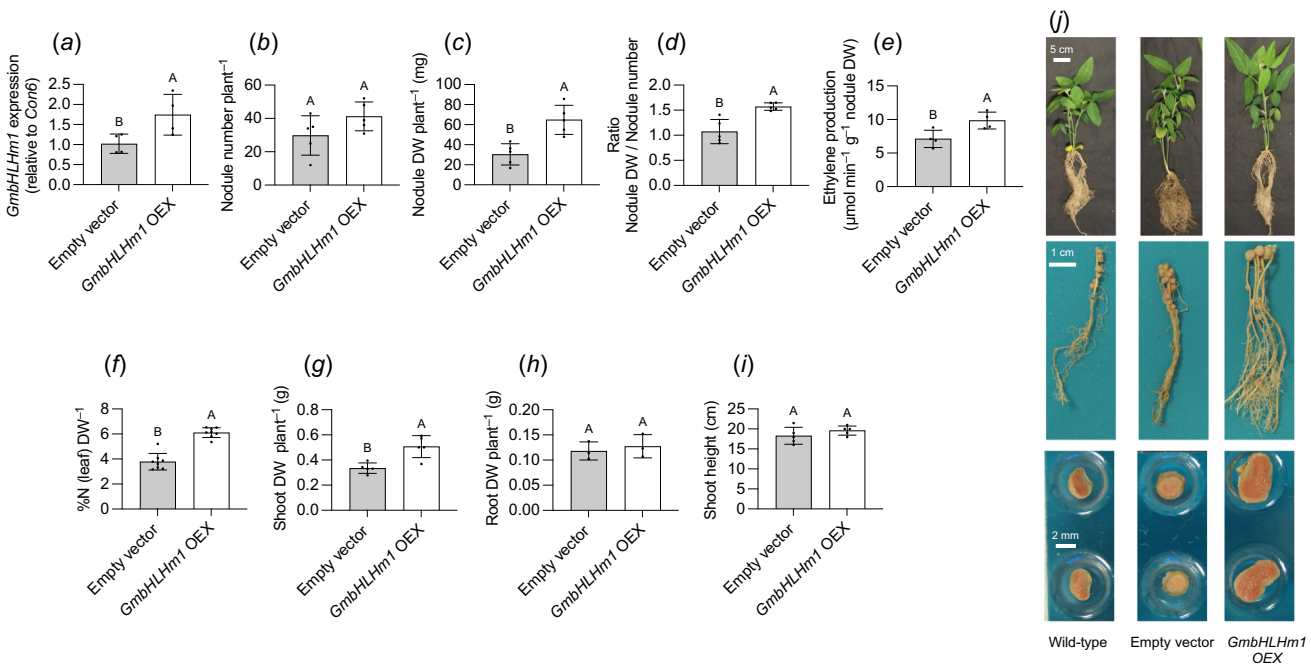

Fig. 3. Overexpression of *GmbHLHm1* in soybean hairy roots. (*a*) Overexpression of *GmbHLHm1* using the *35S* promoter. Changes to nitrogen fixation activities and plant growth in *GmbHLHm1 OEX* and empty vector hairy root soybean plants: (*b*) nodule number per plant root; (*c*) nodule DW per plant root; (*d*) ratio of nodule DW (mg) to nodule number; (*e*) nodule acetylene reduction to ethylene per plant root nodule DW; (*f*) %N (DW) in aerial leaf tissues; (*g*) shoot DW per plant; (*h*) root DW per plant; and (*i*) shoot height at harvest. (*j*) Qualitative and representative images of whole plants, nodulated roots and extracted nodules from wild-type, empty vector, and *GmbHLHm1* OEX lines. The expression of *GmbHLHm1* was normalised with *Con6* as a reference gene and was calculated using the $2^{-\Delta\Delta Ct}$ method (Libault *et al.* 2008). Values were means ± s.d. (n = 3–8 individual plants). Significance was determined using un-paired *t*-tests. Different letters above bars indicate the level of significance ($P < 0.05$).

leaves and an increase in the shoot DW (Fig. 3*d–f*). These positive changes to nodule development and function would suggest *GmbHLHm1* expression and activity are negatively regulated (repressed) in the context of long-term symbiotic N_2-fixation. A prior time course analysis of *GmbHLHm1* expression revealed a strong upregulation of expression at 20 days after rhizobia inoculation (Chiasson *et al.* 2014), which coincides with the development of a matured nodule and the onset of measurable N_2-fixation activity in soybean (Herridge *et al.* 1990; Imsande 1991; Bergersen *et al.* 1992). This enhanced expression in nodules then decreases as the plant transitions to a reproductive growth phase (Chiasson *et al.* 2014). In this experiment, nodule expression of *GmbHLHm1* was evident in the OEX lines at 28 days after rhizobia inoculation, suggesting that constitutive over-expression of *GmbHLHm1* (OEX) supported an extended N_2-fixation capacity, possibly through enhanced nodule development and activities. We are cognisant of the native diel expression pattern previously recorded for *GmbHLHm1* (Chiasson *et al.* 2014) and that our measurement of *GmbHLHm1* expression (taken at 11:00 hours) may actually be an underestimation of its potential level of expression at night.

The mechanisms by which the GmbHLHm1 transcription factor increases nodule growth and activity remains unknown. A range of different genetic markers have been aligned to nodule growth and development. A QTL was identified on Chromosome 11(B1) linked to nodule size and nodule weight (Hwang *et al.* 2014). This was eventually fine mapped to reveal the presence of a cell wall localised β-expansin, *GmINS1* (*INCREASING NODULE SIZE1*) (Li *et al.* 2018). β-expansins belong to a superfamily of Expansin proteins that are involved in pH dependent cell-wall extensions by disrupting hydrogen bonds between cellulose microfibrils and cross-linking glycans (Li *et al.* 2003). Overexpression of *GmINSI* resulted in increased nodule size, nodule weight, and nodule number per plant relative to the empty vector controls. Another cell wall β-expansin, *GmEXPB2*, was identified through soybean Pi starvation assay (Li *et al.* 2015). Like *GmINS*I, overexpression of the nodule localised *GmEXPB2*, increased nodule size and number while suppression of expression reduced nodule development and activity. A third expansin, *GmEXPA11* (Glyma. 04g222100) has recently been linked to nodule enlargement, increased N_2-fixation and N content when overexpressed (Xing *et al.* 2025). Interestingly, *GmEXPA11* is positively regulated through the overexpression of the soybean bHLH transcription factor GmPFT1 (Yang *et al.* 2021; Zhang *et al.* 2024). We have no direct evidence linking expansin activity to *GmbHLHm1* in soybean nodules, but we have previously seen the reduction in expression of an *EXPANSIN A7* (*Glyma. 11g027600*) in soybean roots when *GmbHLHm1* is silenced (Mohammadi Dehcheshmeh 2013), suggesting a possible link in activities. Future research will explore the transcriptional targets of GmbHLHm1 through targeted RNA sequence experiments and DIP-SEQ assays.

The presence of GA responsive elements on the *GmbHLHm1* promoter suggested a putative regulatory pathway linking GmbHLHm1, plant GA and nodule activity. Application of GA_3 to transformed hairy roots expressing *GmbHLHm1Pro:GUS* significantly reduced *GUS* expression and the intensity of visualised GUS signal in both nodules and roots. However, base pair changes to any of the three GA responsive elements individually disrupted *GmbHLHm1Pro:GUS* activity. At this stage it remains unclear what regulatory role the GA elements in the *GmbHLHm1* promoter have on *GmbHLHm1* expression. Nevertheless, the reduction of *GmbHLHm1* expression by short-term GA treatment suggests a nodule-linked responsiveness to GA. For example, exogenous GA_3 application before rhizobia inoculation inhibits nodulation in *Lotus japonicus* through the disruption of infection thread formation (Maekawa *et al.* 2009). Treatment with various GA biosynthesis inhibitors, including chlormequat chloride and uniconazole-P, reduces lateral root-based nodulation in *S. rostrata* in a similar fashion that high levels of applied bioactive GA does (Lievens *et al.* 2005). GA biosynthetic deficient mutants of pea (*Pisum sativum*), show decreased nodulation, while the application of 10^{-6} M GA_3 could restore nodulation to wild-type levels (Ferguson *et al.* 2005). Moreover, low (10^{-9}–10^{-6} M) GA_3 applications can increase nodule numbers in wild-type plants, while increased GA_3 levels (up to 10^{-3} M) decrease nodule numbers in both wild-type and GA deficient mutant peas (Ferguson *et al.* 2005). Akamatsu *et al.* (2021) reported that GA biosynthesis is activated during nodule formation inside the vascular bundles. Chu *et al.* (2022) reported that spatio-temporal changes of GA biosynthesis genes and their distribution in the nodulated root system play an important role during nodulation. A mutation study revealed that both high and low GA concentrations influence GA signalling in GA biosynthesis deficiency mutants of pea (*na*, *ls*, *lh*) and a constitutive GA signalling mutant, results in suppression of nodule formation (Ferguson *et al.* 2005; Ferguson *et al.* 2011). The links between GA and transcription factors was documented through a GA-responsive *cis*-acting region discovered on the *NIN* promoter, indicating endogenous GA may play a central role in coordinating nodule development through NIN activity (Akamatsu *et al.* 2021; Shen and Feng 2024). McAdam *et al.* (2018) suggested that biosynthesised GA promotes nodule organogenesis into N_2-fixing nodules via the activity of the DELLA protein. In *M. truncatula*, GA signalling mediated by DELLA1 decreased bioactive cytokinin (CK) in roots and negatively regulated the Cytokinin Response1 (CRE1)-dependent NF activation, including CK-signalling genes as well as the CK-regulated early nodulation genes (*NODULATION SIGNALLING PATHWAY 2* (*NSP2*) and Ethylene Response Factor Required for Nodulation1) (Fonouni-Farde *et al.* 2016). High levels of GA would have an impact on DELLA protein stability and could influence several GA-responsive transcription factors including *GmbHLHm1*. Recent evidence has revealed that GA helps regulate root endodermal cells promoting lateral root and nodule organogenesis,

as well as help repress rhizobial infection through the induction of secondary signals (Velandia *et al*. 2024). This regulation is most likely linked to cellular GA pools. Using a GA biosensor (GIBERELLIN PERCEPTION SENSOR 2), GA accumulation is identified at the site of nodule primordia in *M. truncatula*, increasing in concentration in nodule cortical cells and finally in the nodule meristem as it matures (Drapek *et al*. 2024).

The GmbHLHm1 TF has a significant impact on nodulation and nodule development in soybean. Reduced activity curtails nodule growth, N_2-fixation and the delivery of reduced N to aerial tissues, while overexpression increases nodule activity (growth and N_2-fixation) while delivering increased N to aerial tissues. This study also suggests a relationship between GA and *GmbHLHm1* expression, possibly acting as a negative regulator. We observed that extended GA treatments had a negative impact on promoter activity. However, we did not observe a direct link to either of the three identified GA-motifs located on the *GmbHLHm1* promoter, where disruption of either motif eliminated promoter activities independent of GA treatment. This suggests that each of these elements may be required to function as a promoter *per se*, while possibly having a yet defined role in a GA signalling cascade. It will be important to further understand if a direct relationship to endogenous GA exists with GmbHLHm1 to help encourage or negate nodule development and function.

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Data availability. All background data (raw data) and DNA constructs will be made available upon request.

Conflicts of interest. The authors declare that they have no competing interests, or other interests that might be perceived to influence the results and/or discussion reported in this paper.

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