



RESEARCH PAPER

GmYUC2a mediates auxin biosynthesis during root development and nodulation in soybean

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Abstract

Auxin plays central roles in rhizobial infection and nodule development in legumes. However, the sources of auxin during nodulation are unknown. In this study, we analyzed the *YUCCA* (*YUC*) gene family of soybean and identified *GmYUC2a* as an important regulator of auxin biosynthesis that modulates nodulation. Following rhizobial infection, *GmYUC2a* exhibited increased expression in various nodule tissues. Overexpression of *GmYUC2a* (*35S::GmYUC2a*) increased auxin production in soybean, resulting in severe growth defects in root hairs and root development. Upon rhizobial infection, *35S::GmYUC2a* hairy roots displayed altered patterns of root hair deformation and nodule formation. Root hair deformation occurred mainly on primary roots, and nodules formed exclusively on primary roots of *35S::GmYUC2a* plants. Moreover, transgenic *35S::GmYUC2a* composite plants showed delayed nodule development and a reduced number of nodules. Our results suggest that *GmYUC2a* plays an important role in regulating both root growth and nodulation by modulating auxin balance in soybean.

Keywords: Auxin, nodulation, nodule development, root development, root hair deformation, soybean.

Introduction

Nodulation in legumes is coordinated by two distinct developmental programs, rhizobial infection and nodule organogenesis. Flavonoids exuded by legume roots induce Nod Factor (NF) production by rhizobia, which are subsequently perceived by root epidermal cells of legume host plants to initiate rhizobial infection and nodule organogenesis (i.e. cell divisions) in the root cortex (Mathesius, 2001; Madsen *et al.*, 2003; Wasson *et al.*, 2006). Rhizobia enter growing root hairs and progress towards

the root cortex through infection threads; simultaneously, some root cortical cells re-enter the cell cycle and contribute to the initiation of the nodule primordia (Ferguson *et al.*, 2010, 2019). These processes eventually converge where rhizobia are released in infection droplets into the dividing cortical cells of the nodule primordia. Each process involves a highly complex gene network; however, little is known about how they are spatially regulated and integrated.

It has long been known that rhizobial infection and root cortical cell divisions are regulated by endogenous cues (e.g. plant hormones) following rhizobial recognition (Ferguson and Mathesius, 2014). For example, auxin plays a central role in nodule organogenesis, similar to its function in lateral roots (Kohlen *et al.*, 2018). Auxin stimulates cortical cell division in response to rhizobial infection, leading to the formation of determinate or indeterminate nodules (Thimann, 1936; Van Noorden *et al.*, 2006; Suzaki *et al.*, 2012). The distribution of auxin and its peak concentration have been studied during nodule primordium formation and development in various legumes, including *Medicago truncatula*, *Lotus japonicus*, and soybean (Pacios-Bras *et al.*, 2003; Bustos-Sanmamed *et al.*, 2013; Turner *et al.*, 2013). However, external application of auxin or inhibitors of auxin biosynthesis or action typically prevents nodule development (Thimann, 1936; Kuppasamy *et al.*, 2009; reviewed in Ferguson and Mathesius, 2003). Thus, auxin gradients and local auxin maxima, which are crucial for nodule initiation and development, are precisely controlled. The auxin response of cortical cells is also altered during nodule initiation and nodule primordium formation (Mathesius *et al.*, 1998; Suzaki *et al.*, 2012). Nodule numbers are regulated by auxin receptors and auxin response factor genes (*ARF* genes), which are targeted by miR160/miR167 and miR393, respectively, with some differences between determinate and indeterminate legume nodule development (Turner *et al.*, 2013; Nizampatnam *et al.*, 2015; Wang *et al.*, 2015; Cai *et al.*, 2017).

Evidence indicates that the distribution and maxima of auxin are regulated mainly by local auxin transport. Early studies indicated that synthetic auxin transport inhibitors such as 2,3,5-triiodobenzoic acid or 1-*N*-naphthylphthalamic acid triggered the formation of nodule-like structures or pseudonodules in the absence of rhizobia in some legumes (Hirsch *et al.*, 1989). This suggests that the inhibition of auxin transport is sufficient to establish local auxin maxima that can activate programs governing nodule initiation and organogenesis. A recent computational study supported the notion that auxin transport is required for nodule initiation and development, and that reduced auxin export plays a dominant role in auxin accumulation at sites of nodule initiation (Deinum *et al.*, 2012). Previously, it was shown that *MtLAX1* and *MtPIN*, which encode auxin import and export proteins, respectively, are expressed during nodulation (de Billy *et al.*, 2001), and silencing *MtPIN* genes reduced nodule numbers (Huo *et al.*, 2006). Recently, *MtLAX2*, which is a paralog of Arabidopsis *AUX1*, was reported to positively regulate nodulation in *M. truncatula* (Roy *et al.*, 2017). These results confirm the importance of auxin transport in nodule development, but how these transporters (especially PIN proteins) are regulated and subcellularly localized in response to rhizobial infection and nodule formation is unknown.

Using *M. truncatula*, Breakspear *et al.* (2014) demonstrated that auxin signaling is also required for rhizobial infection of root hairs, with several auxin-responsive genes, including *ARF16a*, *GH3.1*, *IAA9*, and *SAUR1*, being induced following inoculation. The expression of auxin-responsive marker genes was also shown to be induced by rhizobial infection of

root hairs, and mutation of *ARF16a* impaired early infection events, further indicating that auxin is required for infection. Collectively, this suggests that increased auxin levels and auxin signaling are necessary for rhizobial infection, and, importantly, that the pathway of auxin-mediated rhizobial infection is conserved in both determinate and indeterminate nodulating legumes (Breakspear *et al.*, 2014). Recently, in *Lotus japonicus*, an optimized DII-based auxin accumulation sensor was used, and confirmed the appearance of auxin accumulation in root hairs in response to NF (Nadziejka *et al.*, 2018). The precise source of auxin and the molecular mechanisms controlled by auxin signaling are yet to be fully determined.

An array of Arabidopsis mutants was used to establish that genes involved in auxin metabolic pathways are differentially regulated during various development processes (Ljung, 2013). *YUCCA* (*YUC*) flavin monooxygenases have been discovered in Arabidopsis and confirmed to be responsible for synthesizing indole-3-acetic acid (IAA) from indole-3-pyruvic acid (IPyA) (Mashiguchi *et al.*, 2011; Stepanova *et al.*, 2011; Won *et al.*, 2011), and appear to be involved in the major pathways for IAA biosynthesis in plants (Zhao, 2012). Identification and analysis of *YUC* genes in various species including Arabidopsis (Cheng, 2006), petunia (Tobena-Santamaria *et al.*, 2002), and rice (Yamamoto *et al.*, 2007; Woo *et al.*, 2007) have illustrated their phylogenetic relationship and crucial roles in plant development (Zhao, 2008; Mashiguchi *et al.*, 2011; Poulet and Kriechbaumer, 2017). Changes in expression levels of *YUC* genes have a great impact on auxin biosynthesis, leading to defects in root development (Mashiguchi *et al.*, 2011; Stepanova *et al.*, 2011; Won *et al.*, 2011). For example, the root phenotype of the *Atyuc1D* mutant is similar to that of known auxin overproduction mutants with shorter primary roots and numerous long root hairs (Zhao *et al.*, 2001). Overexpression of *YUCCA2* and *YUCCA3* also causes a similar root phenotype (Zhao *et al.*, 2001). Single or double knockout alleles of *YUCCA* and *YUCCA2* show no obvious phenotypic differences, suggesting that there is likely to be redundancy between their roles in plant development (Zhao *et al.*, 2001).

Here, we report our findings from studies designed to determine whether auxin biosynthesis in roots and root hairs contributes to rhizobial infection and nodule organogenesis in soybean. By initially analyzing publicly available expression data, we identified the differential expression of a large family of *YUCCA* genes in various organs and tissues of soybean plants. In particular, we systematically analyzed the function of *GmYUC2a*, which is highly expressed in soybean nodules. We found that *GmYUC2a* regulates auxin biosynthesis, and its overexpression results in severe growth defects in primary root elongation, lateral root development, and root hair elongation. Our results further revealed that *GmYUC2a* is responsive to rhizobial infection, and its promoter exhibited strong activity in maturing nodules; additionally, *GmYUC2a* mediates not only early nodule development, but also nodule senescence. Collectively, our findings demonstrate that *GmYUC2a*-mediated local auxin biosynthesis contributes to rhizobial infection and nodule organogenesis, including nodule senescence, in soybean nodules.

Materials and methods

Phylogenetic analysis

All sequences were downloaded from Phytozome (<https://phytozome.jgi.doe.gov/pz/portal.html>). Using ClustalX 1.83 (Thompson *et al.*, 1997) and MEGA6.0 (Tamura *et al.*, 2011), a phylogenetic tree was constructed based on the amino acid sequences (<http://phytozome.jgi.doe.gov/pz/portal.html>) of the homologous proteins of AtYUC family members in soybean and *M. truncatula* with 1000 bootstraps.

Gene structure analysis

Exon–intron structure information for *GmYUC* genes was obtained using Phytozome v12.0. A gene structure schematic diagram was made using the Gene Structure Display Server (Guo *et al.*, 2007).

Heatmaps

Expression data for all *GmYUC* genes were collected from SoyBase (<http://soybase.org/soyseq/>; Severin *et al.*, 2010). Heatmap Illustrator (v1.0) was used to generate heatmaps of differentially expressed *GmYUC* genes (Deng *et al.*, 2014).

Plant materials and growth conditions

Soybean (*Glycine max* [L.] Merrill) cv Williams 82 plants were grown in a greenhouse (16 h of light/8 h of dark; 25 °C; 50% relative humidity) in vermiculite and irrigated with a nitrogen-deficient solution as described by Wang *et al.* (2009). *Bradyrhizobium diazoefficiens* USDA110 was used for all nodulation experiments (OD₆₀₀=0.08). To analyze gene expression in response to rhizobial infection, roots of 10-day-old plants were inoculated with 30 ml of a suspension of *B. diazoefficiens*. The plants were watered every 7 d, alternating between a nitrogen-deficient nutrient solution and water. Different soybean tissues were harvested at specified time points after inoculation. For root samples, the de-nodulated roots were used for real-time quantitative reverse transcription PCR (RT–qPCR) assays.

RNA extraction and RT–qPCR

To monitor transcript levels of *GmYUC2a*, total RNA was extracted from leaves, roots, and nodules using Trizol reagent (Tiangen Biotech [Beijing] Co. Ltd, Beijing, China), and 2 µg of total RNA treated with DNase I (Invitrogen, Carlsbad, CA, USA) were used to synthesize the first cDNA strand with a FastQuant RT Kit (Tiangen Biotech [Beijing] Co. Ltd). RT–qPCR was performed using SuperReal PreMix Plus (SYBR Green; Tiangen Biotech [Beijing] Co. Ltd) with gene-specific primers. The housekeeping gene *GmELF1b* was used as a reference (Jian *et al.*, 2008), and relative expression was measured using the 2^{–ΔΔCT} method (Livak and Schmittgen, 2001). All primers used in this study are listed in Supplementary Table S3 at JXB online. In order to ensure the primer specificity of *GmYUC2a*, *GmYUC2b*, and *GmYUC2c* used for RT–qPCR, the products were amplified via PCR and then ligated to the T-Vector pMD-19 (Takara Bio Inc. Code No. 3271) for sequencing. All primers used in qRT–PCR are included in Supplementary Table S3.

Vector construction

To generate the promoter::GUS (β-glucuronidase) reporter fusion construct of *GmYUC2a*, the genomic region upstream of the *GmYUC2a* coding sequence (1467 bp) was amplified by PCR from Williams 82 genomic DNA and cloned upstream of the *GUS* coding sequence in pCAMBIA3301 using *Bam*HI and *Bgl*II. To make the overexpression construct of *GmYUC2a*, the coding sequence of *GmYUC2a* (1248 bp) was amplified by PCR using KOD DNA polymerase (Toyobo, Osaka, Japan) from Williams 82 cDNA and inserted into the binary vector pTF101–GFP directly under the control of the *Cauliflower mosaic virus* 35S promoter (35S) sequence using *Sma*I and *Bam*HI. To make the RNAi construct, a 122 bp segment of the *GmYUC2a* coding sequence was

amplified from Williams 82 cDNA by PCR and cloned into pTCK303 using *Kpn*I/*Spe*I and *Bam*HI/*Sac*I in the sense and antisense orientations. Gene-specific primers are provided in Supplementary Table S3.

DNA extraction and characterization of transgenic roots

DNA was extracted from hairy root samples using a modified version of the cetyltrimethylammonium bromide (CTAB) method (Porebski *et al.*, 1997). The *Bar* or *HygromycinB* (*Hyg*) gene was used for molecular validation of the transformed hairy roots according to the selective marker in the binary vectors utilized for *Agrobacterium rhizogenes*–mediated hairy root transformation (Kereszt *et al.*, 2007). The *Bar*– and *Hyg*–specific primers used are listed in Supplementary Table S3.

Soybean hairy root transformation and subsequent

B. diazoefficiens inoculation

Soybean hairy root transformation was performed as described previously with modifications (Kereszt *et al.*, 2007; Jian *et al.*, 2009; Wang *et al.*, 2014). *Agrobacterium rhizogenes* strain K599 was used for transformation. Transgenic composite plants were transferred to pots (13 cm×10 cm×8.5 cm) containing vermiculite and watered with nitrogen-deficient nutrient solution, grown for 7 d (16 h of light/8 h of dark; 25 °C; 50% relative humidity), and then inoculated with 30 ml of *B. diazoefficiens* USDA110 (OD₆₀₀=0.08) per plant.

Root hair deformation and nodulation assays

To analyze root hair deformation and nodule formation, transgenic roots were collected and stained with methylene blue. Root hair deformation was analyzed under a light microscope at 3, 6, and 10 days after inoculation (DAI) as described previously (Wang *et al.*, 2014). Nodule numbers were determined at specified time points after rhizobial inoculation.

Histochemical localization of GUS expression

To examine tissue- and cell-specific promoter activity of *GmYUC2a*, the *GUS* reporter gene was expressed under the control of the *GmYUC2a* promoter (1467 bp located directly upstream of *GmYUC2a*), and transgenic roots and nodules were stained as described previously for the histochemical localization of GUS expression (Jefferson *et al.*, 1987).

Auxin response assay

For gene expression analysis in response to exogenous auxin application, soybean seeds (cv Williams 82) were germinated in vermiculite irrigated once with a low-nitrate solution (Wang *et al.*, 2009). Seven-day-old seedlings were treated with or without 1 µM 2,4-dichlorophenoxyacetic acid (2,4-D) or 1-naphthaleneacetic acid (NAA) (Turner *et al.*, 2013; Wang *et al.*, 2015). 6-Benzyladenine (6-BA; 5 µM) was used as a control for analyzing the expression of *GmNIN*. After 24 h, the roots were collected and used for analyzing gene expression. For phenotypic analysis of hairy roots in response to 2,4-D, composite plants harboring K599 were transplanted into vermiculite and watered with the low-nitrate solution containing different concentrations of 2,4-D (0, 0.2, and 0.4 µM). Ten days after transplanting, the hairy root phenotypes were noted and root length measured.

Imaging and histological analysis of nodules

Nodules from transgenic roots were analyzed using an Olympus microscope (SZ2-ILST; Olympus Corp., Tokyo, Japan). To produce transverse and longitudinal sections, roots and nodules at different stages after inoculation were collected and fixed in FAA solution, and then dehydrated in a graded alcohol series (Charon *et al.*, 1997). Nodule sections 25 µm thick were produced using a vibratome (VT1200S; Leica, Wetzlar, Germany), mounted between a slide and coverslip, and observed with an Olympus microscope (CX31; Olympus Corp., Tokyo, Japan).

Measurement of IAA

To determine the IAA content, transgenic hairy roots harboring an empty vector (EV) or the 35S::*GmYUC2a* construct were collected 10 d after transplantation. IAA was extracted and the IAA content was measured using LC–tandem MS as described by Fu *et al.* (2012) and Sun *et al.* (2015).

Statistical analysis

Data were analyzed using Student's *t*-tests or one-way ANOVAs with SigmaPlot 10.0 or GraphPad Prism 5. Statistically significant differences are marked in the figures with '***' ($P < 0.001$) or 'ns' (not significant; $P > 0.05$). Different letters were used to indicate significant differences by the Student–Newman–Keuls test ($P < 0.05$).

Results

Phylogenetic analysis of YUCCA family genes in *Arabidopsis*, soybean, and *M. truncatula*

YUCCA proteins regulate the limiting step of auxin biosynthesis. To identify the YUCCA gene family of soybean and *M. truncatula*, BLAST searches of the *Glycine max* (v2.0) and *M. truncatula* (Mt4.0 v1) genomes were conducted using protein sequences of 11 Arabidopsis YUCCA-encoding genes (www.arabidopsis.org). Fourteen putative MtYUC- and 22 putative GmYUC-encoding loci were identified based on the assigned nomenclature/classification, and each showed high sequence similarity (Supplementary Fig. S1; Supplementary Table S1).

The soybean genome has experienced duplication and diploidization (Schmutz *et al.*, 2010). To analyze the evolutionary relationships of YUCs among Arabidopsis, soybean, and *M. truncatula*, a phylogenetic tree was constructed, which was generated in MEGA6.0 with 1000 bootstraps using amino acid sequences (Supplementary Dataset S1). Based on protein similarity and the phylogenetic tree (Supplementary Fig. S1A; Supplementary Table S1), the soybean YUC proteins were named according to the YUC family cluster from Arabidopsis. As shown in Supplementary Fig. S1A, the GmYUCs were divided into five subfamilies: GmYUC5/8/9 (GmYUC5–8–9a/b/c/d), GmYUC3/7 (GmYUC3–7a/b), GmYUC2/6 (GmYUC2a/b/c/d, GmYUC6a/b/c/d), GmYUC1/4 (GmYUC1–4a/b/c/d), and GmYUC10/11 (GmYUC10a/b, GmYUC11a/b). The homologous YUC family genes in *M. truncatula* were more closely related to those of soybean than those of Arabidopsis (Supplementary Fig. S1A), consistent with them both being legumes. To further understand their evolutionary relationship, the gene structures were analyzed with the Gene Structure Display Server using genomic and coding sequences (Supplementary Dataset S1). Strong conservation between the gene structures was found, with each composed of four exons and three introns (Supplementary Fig. S1B).

GmYUC genes show divergent expression patterns in soybean

Given that the *GmYUC* gene family is large and each *GmYUC* gene has a variable number of duplicates, we speculated that

GmYUC genes play divergent roles in the control of auxin biosynthesis in a tissue-/organ-specific pattern to modulate plant growth and environmental responses. To explore the expression patterns of *GmYUC* genes, we analyzed the expression of 22 *GmYUC* genes in different tissues, mainly including young leaf, flower, pod, pod shell, root, nodule (20–25 d after rhizobial inoculation), and seeds collected at different developmental stages, using RNA sequencing (RNA-Seq) data from SoyBase (<http://soybase.org/>; Severin *et al.*, 2010). Interestingly, nearly all *GmYUC* genes were expressed at low levels in the tissues/organs examined, yet in many cases their mRNAs were barely detected (Supplementary Fig. S2; Supplementary Table S2). Six of the genes, *GmYUC2a/b/c*, *GmYUC5*, *GmYUC6d*, and *GmYUC11d* were expressed in nodules, with *GmYUC2a* being the most highly expressed (Supplementary Fig. S2; Supplementary Table S2). Hence, our findings do suggest divergent expression patterns for the *GmYUC* genes.

GmYUC2a expression during rhizobial inoculation and nodule organogenesis

To verify the nodule-specific expression of *GmYUC2a*, *GmYUC2a* expression was determined in leaves, roots (de-nodulated), and nodules at 10, 17, and 28 DAI with *B. diazoefficiens*. As shown in Fig. 1A, *GmYUC2a* was much more highly expressed in nodules than in leaves and roots at 10 d or 17 d after rhizobial infection, and the expression of *GmYUC2a* was highest in the nodules at 17 DAI compared with 10 and 28 DAI (Fig. 1A). This suggests a crucial role for *GmYUC2a* in soybean nodulation. Using an inoculation time-course study, *GmYUC2a* expression was found to reach its peak at 3 DAI (Fig. 1B), then rapidly decreased down to its pre-infection level by 5 DAI, where it remained throughout subsequent stages of nodule development (Fig. 1B). Thus, *GmYUC2a* may act to modulate rhizobial infection and early nodule formation.

To examine the tissue-specific promoter activity of *GmYUC2a* during nodulation, we generated transgenic hairy roots expressing *GmYUC2a pro::GUS*. Interestingly, in emerging nodules at 10 DAI, the promoter of *GmYUC2a* showed uniformly higher activity in all nodule tissues (Fig. 1C, D) and, in nodules close to maturation at 28 DAI, *GmYUC2a* was observed to have high promoter activity in the cortex and infection zone/nitrogen fixation zone (Fig. 1E, F). Finally, in older nodules near senescence, *GmYUC2a* promoter activity was substantially decreased, with GUS activity restricted to the vascular bundles of nodules (Fig. 1G, H). Taken together, these data suggest that *GmYUC2a* might be involved in root development, rhizobial infection, nodule organogenesis, and nodule senescence.

GmYUC2a overexpression dramatically affects the growth of roots and root hairs

To functionally characterize *GmYUC2a*, we generated transgenic composite plants overexpressing *GmYUC2a* under the control of the 35S promoter (Supplementary Fig. S3A). Phenotypic analysis showed that root hairs of 35S::*GmYUC2a*

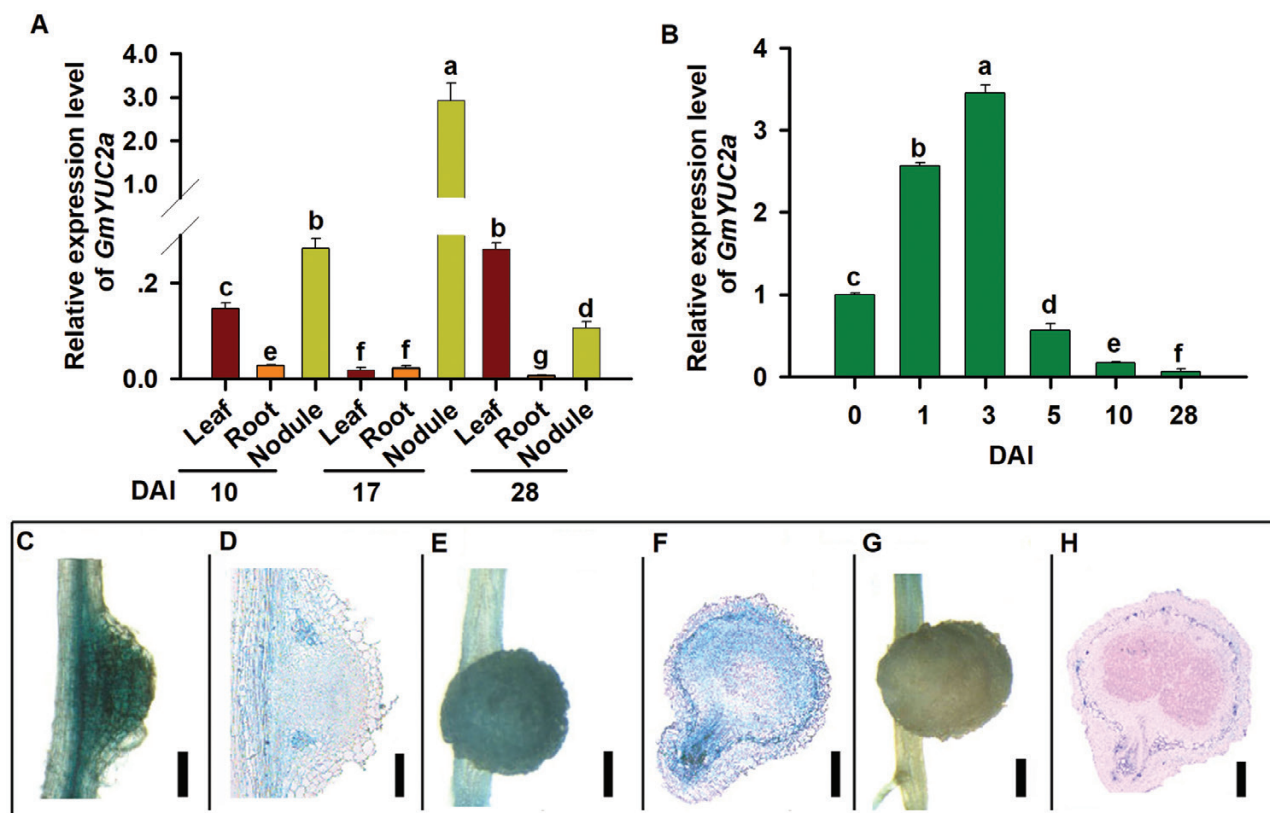


Fig. 1. The expression pattern of *GmYUC2a* in different tissues and in response to rhizobial infection. (A) The expression of *GmYUC2a* in leaves, roots (de-nodulated), and nodules at 10, 17, and 28 DAI with *B. diazoefficiens*. (B) *GmYUC2a* expression in roots at 0, 1, 3, 5, 10, and 28 DAI. *GmELF1b* was used as a reference gene to determine relative expression. All experiments consisted of three independent biological replicates. Root sections 4 cm from the root tip were collected from six plant samples and were used in each biological replicate. Data are the average of three biological repeats. Error bars indicate the SE. Different letters indicate significant differences by the Student–Newman–Kuels test ($P < 0.05$). (C–H) GUS activity in *B. diazoefficiens*-inoculated transgenic roots and nodules (C, 10 DAI, scale bar=600 μ m; D, 10 DAI, scale bar=300 μ m; E, 28 DAI, scale bar=600 μ m; F, 28 DAI, scale bar=800 μ m; G, 55 DAI, scale bar=1 mm; H, 55 DAI, scale bar=1 mm). (This figure is available in color at JXB online.)

transgenic roots were significantly longer than those of control roots, and the root hair density was shown to be clearly increased (Fig. 2A, B; Supplementary Fig. S3B). Notably, severe defects in root growth were also observed in *35S::GmYUC2a* plants. The length of *35S::GmYUC2a* roots was reduced by up to 80% (Fig. 2C, D) and the lateral root number was shown to be clearly reduced by ~75.6% (Fig. 2E). The apical meristem of *35S::GmYUC2a* roots became thinner, whereas the root elongation and maturation zones were significantly thicker than vector-only control roots (Fig. 2F–H). These root phenotypes of the *35S::GmYUC2a* lines mirrored those of Arabidopsis plants overexpressing *YUCCA*, which overproduce auxin (Zhao *et al.*, 2001). These results indicate that *GmYUC2a* has a similar function to Arabidopsis *YUCCA* genes in auxin biosynthesis. To confirm this, we ectopically expressed *GmYUC2a* in Arabidopsis ecotype Col-0 plants and found that this caused a severe short root phenotype (Supplementary Figs. S4A–C), in agreement with previous Arabidopsis results (Zhao *et al.*, 2001). In addition, the hairy roots harboring the EV showed a similar phenotype in response to exogenous auxin (2,4-D) treatment (Supplementary Figs. S5A, B). Together, these data suggests that *GmYUC2a* may modulate root growth by stimulating auxin biosynthesis.

GmYUC2a overexpression increases auxin biosynthesis and enhances the auxin sensitivity of infected roots

To test whether *GmYUC2a* controls auxin biosynthesis in soybean, we first measured the auxin content of *A. rhizogenes*-mediated hairy roots. As shown in Fig. 3A, the auxin content was markedly increased in *35S::GmYUC2a* roots compared with EV control roots at 10 d after transplantation, suggesting that *GmYUC2a* positively regulates auxin synthesis in soybean. Next, we ectopically overexpressed *GmYUC2a* in wild-type Arabidopsis Col-0 plants expressing *DR5::GUS*, which can be used to detect visually the endogenous level and spatial distribution of auxin (Ulmasov *et al.*, 1997). As shown in Fig. 3B and C, the roots of the *DR5::GUS* lines overexpressing *GmYUC2a* were significantly thicker than those of the control plants, indicating that *GmYUC2a* was successfully overexpressed. In *DR5::GUS* lines co-transformed with EV, GUS activity was mainly observed in root tips (Fig. 3C). In sharp contrast, GUS activity was strong and uniformly distributed in all cells/tissues of *35S::GmYUC2a* transgenic roots (Fig. 3C). These results demonstrate that *GmYUC2a* is a functional soybean ortholog of *AtYUC2* that controls auxin biosynthesis. To determine

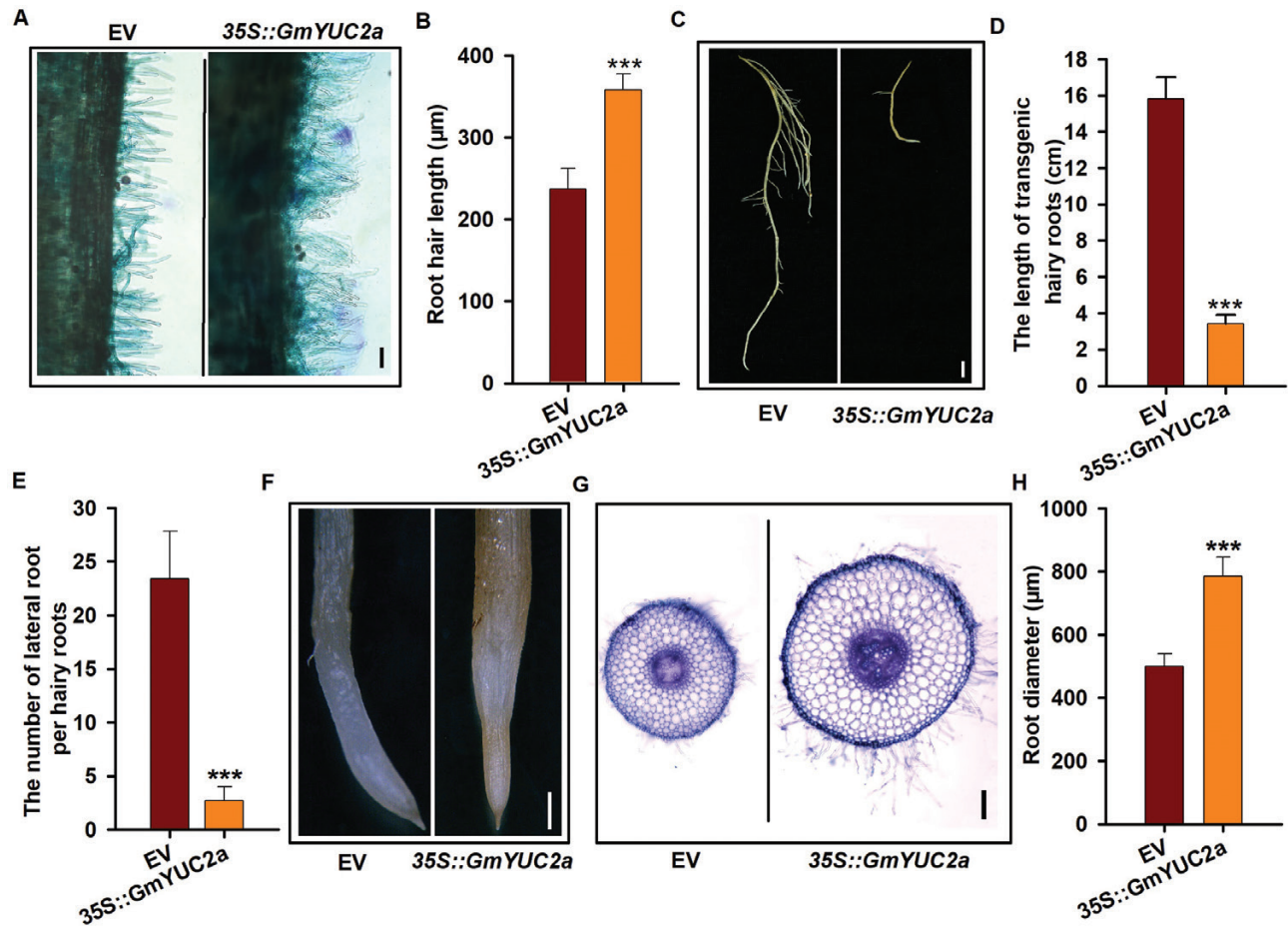


Fig. 2. *GmYUC2a* regulates the growth of roots and root hairs. (A, B) The root hair phenotype (A, scale bar=100 μm) and root hair length (B) in *35S::GmYUC2a* transgenic roots and the EV control at 10 d after transplantation. (C, D) EV and *35S::GmYUC2a* hairy roots at 10 d after transplantation (C, scale bar=5 mm). (E) The number of lateral roots in the transgenic hairy roots harboring EV and *35S::GmYUC2a*. (F–H) Comparison of the root diameter of maturation zones between *35S::GmYUC2a* and EV transgenic roots at 10 d after transplantation (F, scale bar=500 μm); (G) cross-section of transgenic roots harboring *35S::GmYUC2a* or EV and stained with methylene blue (scale bar=100 μm); (H), diameters of *35S::GmYUC2a* and EV transgenic roots. All values are the averages ±SD from three independent experiments. Three independent biological replicates were performed using 15 hairy roots per replicate. Student's *t*-tests were used to determine significance: ****P*<0.001. (This figure is available in color at JXB online.)

whether *GmYUC2a* affects plant responses to auxin, we analyzed the expression of key auxin signaling genes in transgenic hairy roots. Transcript levels of *GmTIR1C*, *GmARF8a*, and *GmIAA1/14/20* were significantly up-regulated in *35S::GmYUC2a* transgenic roots compared with EV control roots (Fig. 3D–H). Taken together, these results demonstrate the crucial role of *GmYUC2a* in auxin biosynthesis and plant responses to auxin in soybean.

GmYUC2a regulates root hair deformation in response to rhizobial infection

Root hairs represent the main sites of rhizobial infection (Madsen et al., 2003). In order to investigate whether *GmYUC2a* is involved in root hair infection during early nodulation, a thorough analysis of root hair deformation was conducted. Few deformed root hairs were found either in the primary or lateral hairy roots of the *35S::GmYUC2a* composite plants in the condition without rhizobial inoculation (Supplementary Figs S6; S7A–C). In contrast, upon

rhizobial infection, deformed root hairs were mainly observed on lateral roots of EV control roots, peaking at 6 DAI (Fig. 4A–C). This is consistent with our observation that most nodules formed on these lateral roots. Intriguingly, there was a sharp contrast between the numbers of deformed root hairs in *35S::GmYUC2a* and the vector control during infection. At 3 DAI, the numbers of deformed root hairs in primary roots and lateral roots in the hairy roots harboring *35S::GmYUC2a* were similar to those harboring the EV. However, the *35S::GmYUC2a* hairy roots exhibited a significantly lower number of deformed root hairs compared with the EV control roots. (Fig. 4A; Supplementary Fig. S7A). Surprisingly, the number of deformed root hairs in the primary roots of *35S::GmYUC2a* plants increased substantially at 6 DAI, although the total number of deformed root hairs of *35S::GmYUC2a* roots was still lower than that of the EV control roots (Fig. 4B; Supplementary Fig. S7B). At 10 DAI, the total number of deformed root hairs in primary roots of *35S::GmYUC2a* plants was comparable with that of vector control roots, and the number of deformed root hairs in the

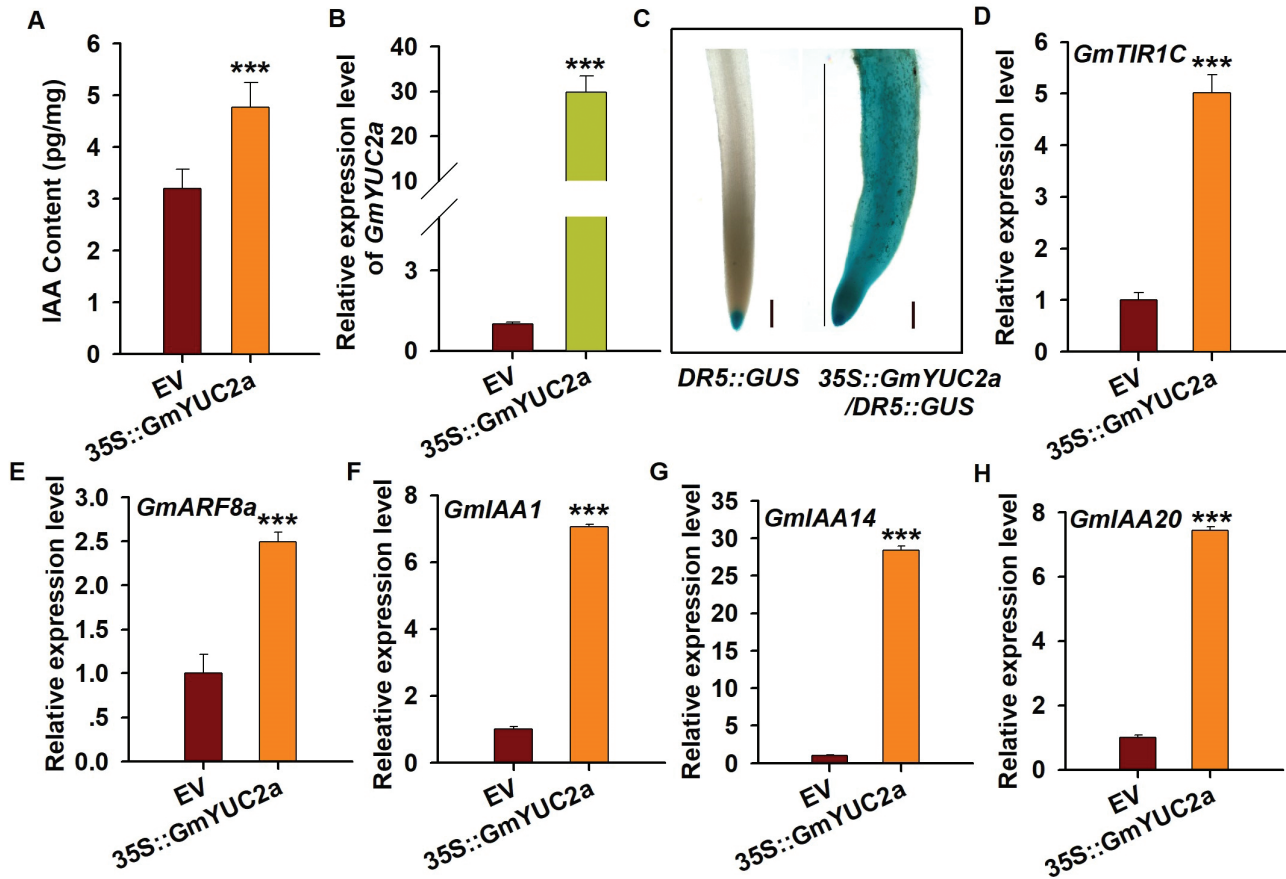


Fig. 3. *GmYUC2a* overexpression increases the auxin content of transgenic roots and regulates the expression of auxin-responsive genes. (A) Measurement of the IAA content in 35S::*GmYUC2a* and EV transgenic roots at 10 d after transplantation. (B) The detection of *GmYUC2a* expression in EV and 35S::*GmYUC2a* transgenic hairy roots. (C) Histochemical staining of GUS activity in *DR5::GUS* and *DR5::GUS* overexpressing *GmYUC2a* transgenic Arabidopsis roots at 10 d after transplantation. Scale bar=500 μ m. (D–H) The transcript levels of *GmTIR1C*, *GmARF8a*, and *GmIAA* genes in EV and 35S::*GmYUC2a* transgenic hairy roots. The gene expression levels were normalized against the geometric mean of the soybean reference gene *GmELF1b*. Three independent biological replications were performed and five transgenic hairy roots showing overexpression of *GmYUC2a* were used per replicate. All values are the averages \pm SD from three independent experiments. Statistically significant differences (Student's *t*-test) are indicated as *** P <0.001. (This figure is available in color at *JXB* online.)

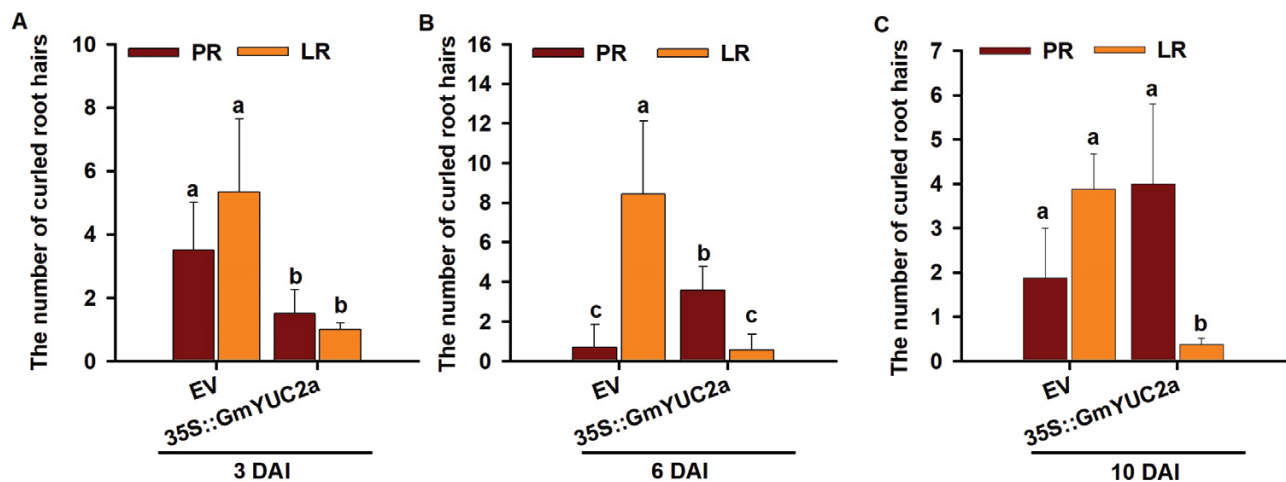


Fig. 4. *GmYUC2a* regulates root hair deformation in response to rhizobial infection. (A–C) *GmYUC2a* overexpression reduced the numbers of deformed and curled root hairs on the primary root (PR) and lateral roots (LR) at 3, 6, and 10 DAI. Segments (2 cm) of hairy roots overexpressing *GmYUC2a* below the root–hypocotyl junction were cut and stained with 1% (w/v) methylene blue. Considerably curled root hairs were counted ($n=10$ –12 hairy roots). All values are the averages \pm SD from three independent experiments. Different letters indicate significant differences by Student–Newman–Kuels test (P <0.05). (This figure is available in color at *JXB* online.)

lateral roots of *35S::GmYUC2a* composite plants remained low (Fig. 4C; Supplementary Fig. S7C). These observations suggest that the levels of *GmYUC2a* expression and auxin may positively regulate rhizobial infection of root hairs.

GmYUC2a overexpression delays nodule organogenesis

To further investigate whether *GmYUC2a* modulates nodule organogenesis, we monitored nodule development in *35S::GmYUC2a* transgenic roots. At 10 DAI, there were ~20

nodules on the EV control lateral roots (Fig. 5A–C). In contrast, no lateral roots or nodules were observed on the primary roots of *35S::GmYUC2a* plants (Fig. 5A, B; Supplementary Fig. S8A). However, longitudinal root sections revealed that nodule primordia had formed on the primary roots of plants expressing *35S::GmYUC2a* (Fig. 5C).

At 17 DAI, the EV control roots continued to exhibit ~20 mature nodules each (Fig. 5D–F; Supplementary Fig. S8B). In contrast, the nodules on the *35S::GmYUC2a* roots were just starting to emerge, densely packed and restricted to the primary nodulation zone (Fig. 5D–F). These nodules matured in

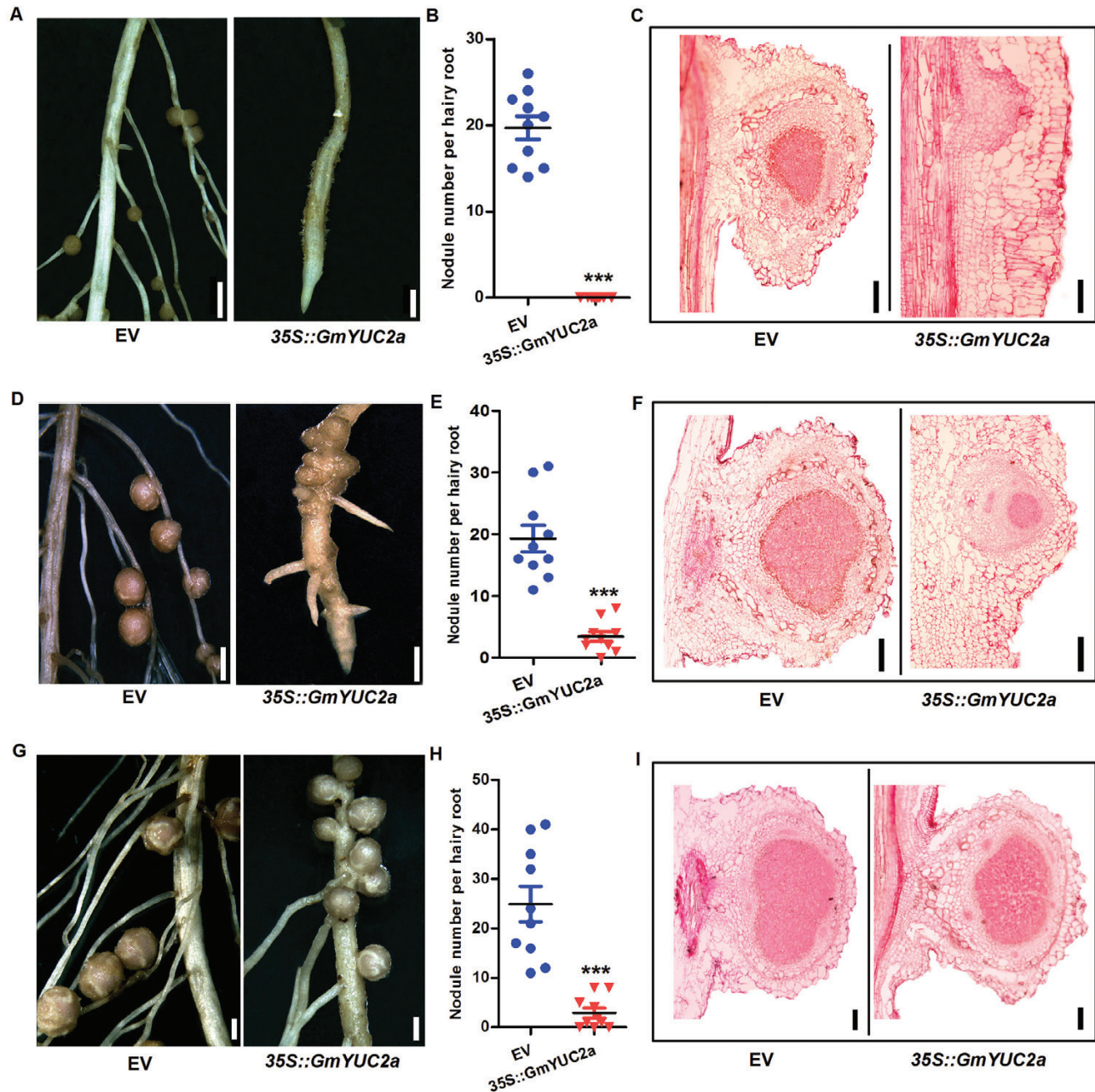


Fig. 5. *GmYUC2a* overexpression delays nodule organogenesis. (A–I) Nodules of individual hairy roots expressing the EV or *35S::GmYUC2a* at 10, 17, and 28 DAI. (A) Scale bar=1500 μ m; (D) EV, scale bar=1500 μ m; *35S::GmYUC2a*, scale bar=1000 μ m; (G) scale bar=1500 μ m. (B, E, H) Quantitative analysis of the nodule number per hairy root expressing the EV and *35S::GmYUC2a* ($n=10$ –12 hairy roots). All values are the averages \pm SD from three independent experiments. Statistically significant differences (Student's *t*-test) are indicated as *** $P<0.001$. (C, F, I) Transverse nodule sections stained with safranin O from EV control and *35S::GmYUC2a* plants. (C) EV, scale bar=150 μ m; *35S::GmYUC2a*, scale bar=100 μ m; (F) EV, scale bar=250 μ m; *35S::GmYUC2a*, scale bar=150 μ m; (I) EV, scale bar=350 μ m; *35S::GmYUC2a*, scale bar=350 μ m. (This figure is available in color at JXB online.)

appearance by 28 DAI (Fig. 5G–I; Supplementary Fig. S8C), indicating that overexpression of *GmYUC2a* and subsequent increased auxin biosynthesis may have delayed nodule development. In particular, we also estimated the ratio of the number of primary root nodules to that of total nodules at 17 DAI. As shown in Supplementary Fig. S8D, overexpression of *GmYUC2a* resulted in the significant increase of nodule numbers appearing in the primary roots.

Next, we generated composite transgenic plants with reduced *GmYUC2a* expression using RNAi, and evaluated their root and nodule development. Neither root nor nodule development was significantly affected (Supplementary Fig. S9A–C) by a reduction in *GmYUC2a*, indicating the redundancy of *GmYUC* genes in the regulation of soybean nodulation, consistent with observations made in Arabidopsis where *AtYUC* genes can function redundantly with one another to regulate root development (Cheng, 2006; Cheng *et al.*, 2007).

Overexpression of *GmYUC2a* regulates rhizobial infection and nodule development through modulating *GmNIN* expression

Nodule Inception (NIN) has been confirmed to be a master regulator of rhizobial infection and nodule organogenesis in legume (Schäuser *et al.*, 1999; Marsh *et al.*, 2007; Griesmann *et al.*, 2018). To test whether *GmYUC2a* overexpression affects nodulation via the *NIN*-mediated signaling pathway, we analyzed the expression patterns of *GmNIN*. Interestingly, qPCR revealed that the expression level of *GmNIN* was clearly increased in the hairy roots harboring *35S::GmYUC2a* in the absence or presence of rhizobia (Fig. 6A, B). Based on this result, we tried to analyze whether *GmNIN* could be induced by exogenous auxin. As shown in Fig. 6C, 6-BA was used as a positive control and it was found to increase the expression level of *GmNIN*. Furthermore, *GmNIN* expression was stably

induced in response to 2,4-D and NAA at 24 h after treatment. These data indicate that *GmYUC2a* has a conserved role in auxin synthesis and might regulate rhizobial infection and the development of nodules by modulating *GmNIN*.

Discussion

Auxin is a central regulator of plant growth and is essential for root development (Benkova and Bielach, 2010; Marhavy *et al.*, 2013). Decades of research have shown that auxin is required for rhizobial infection and nodule organogenesis (de Billy *et al.*, 2001; Ferguson and Mathesius, 2003, 2014; Huo *et al.*, 2006; Roy *et al.*, 2017), but it is not well understood how auxin is synthesized and regulated in the nodule. Here, we show that *GmYUC2a* of soybean is induced by rhizobia and expressed in maturing nodules; it functions in auxin biosynthesis to regulate root growth and nodule organogenesis. These results demonstrate that local auxin biosynthesis is critical for legume nodulation.

YUC genes encode flavin monooxygenases, which catalyze the last step of IAA biosynthesis (IPyA to IAA) in the indole-3-pyruvate pathway (Zhao *et al.*, 2001). Prior to our work, no *YUC* gene had been functionally characterized in soybean. The soybean *YUC* gene family is composed of 22 members that can be divided into four subgroups. Our findings demonstrate that overexpression of *GmYUC2a*, a putative ortholog of Arabidopsis *YUC2* which catalyzes the conversion of IPyA to IAA (Mashiguchi *et al.*, 2011; Stepanova *et al.*, 2011), elevated the auxin content of the root and caused stunted root growth with enhanced root hair elongation. Ectopic overexpression of *GmYUC2a* in Arabidopsis produced growth defects that were similar to those of *AtYUC2*-overexpressing plants (Zhao *et al.*, 2001), indicating that *GmYUC2a* is a functional ortholog of Arabidopsis *YUC2*.

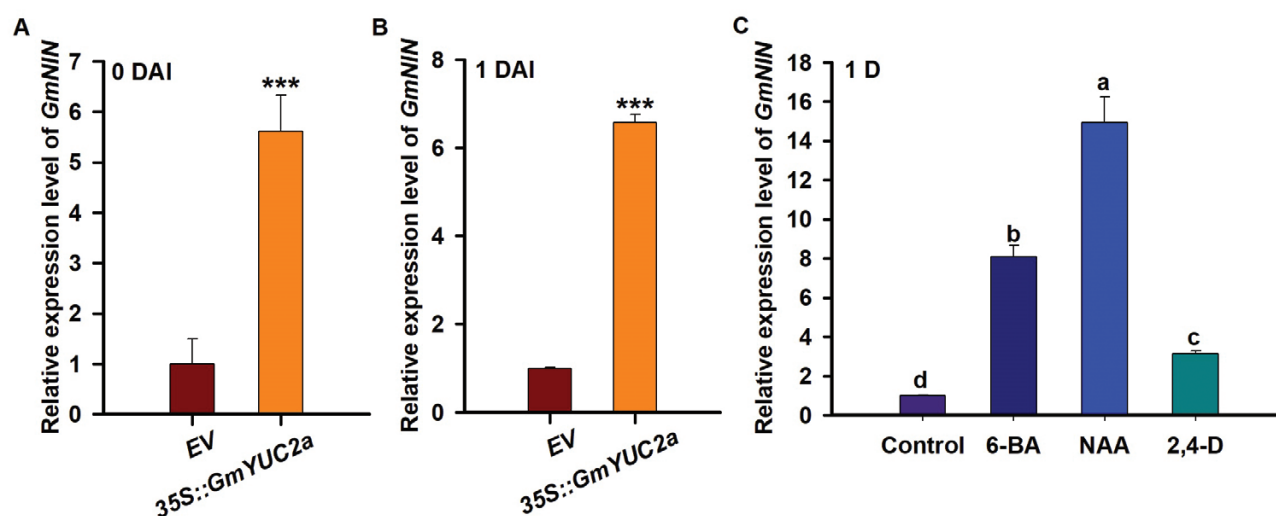


Fig. 6. *GmYUC2a* overexpression and exogenous auxin induce the expression of *GmNIN*. (A and B) *GmNIN* expression in *35S::GmYUC2a* transgenic roots inoculated with *B. diazoefficiens* USDA110. (C) *GmNIN* expression in roots treated with 6-BA, NAA, 2,4-D, or an equal volume of distilled water (control) for 24 h. Three independent biological replications were performed; the gene expression levels were normalized against the geometric mean of the soybean reference gene *GmELF1b*. All experiments were conducted three times. Error bars indicate the SD. Statistically significant differences (Student's *t*-test) are indicated as *** $P < 0.001$. (This figure is available in color at JXB online.)

Previous studies have highlighted a role for auxin in nodule primordium formation and rhizobial infection (e.g. Hirsch *et al.*, 1989; Wasson *et al.*, 2006; Rightmyer and Long, 2011; Turner *et al.*, 2013; Breakspear *et al.*, 2014). Computational and pharmacological data indicate that auxin transport may be a major contributor to the increase in auxin during nodule primordium formation (Deinum *et al.*, 2012). Genetic evidence has also been reported by Roy *et al.* (2017). However, direct evidence for the source of that auxin is lacking, and there is no report on the involvement of auxin biosynthesis in legume nodulation. Here, we showed that *GmYUC2a* was expressed in soybean roots and the maturing nodules of soybean. Taking into account the functions of *GmYUC2a* in root development and root hair elongation, it is apparent that *GmYUC2a* plays a conserved role in root growth regulation. Interestingly, *GmYUC2a* overexpression reduced the number of deformed root hairs, which occurred mainly on the primary roots. Our results suggest that although *GmYUC2a* is an important regulator, it is not the only YUC gene to modulate auxin biosynthesis during rhizobial infection. Indeed, knockdown of *GmYUC2a* did not affect nodulation, and two additional *GmYUC* genes were found to be highly expressed in soybean nodules based on RNA-Seq data. Since both the gradient and maxima of auxin are important for cell growth and differentiation, it is conceivable that auxin biosynthesis is dynamically controlled.

Local increases in auxin in the pericycle and surrounding cells control lateral root initiation (Benkova and Bielach, 2010; Marhavy *et al.*, 2013), whereas increases in cortical cells are reported to be important for nodule initiation (Suzaki *et al.*, 2012). Our results show that local auxin biosynthesis catalyzed by *GmYUC2a* plays an important role in nodule organogenesis. Upon rhizobial infection, *GmYUC2a* promoter activity was uniformly strong in all tissues of maturing nodules, but was only expressed in vascular tissue of mature nodules. We therefore propose that the role of *GmYUC2a*-mediated auxin biosynthesis in nodule development is in cell elongation and nodule expansion, as has been demonstrated for auxin in other tissues (Perrot-Rechenmann, 2010). The disappearance of *GmYUC2a* from the nitrogen fixation zone of senescing nodules suggests that local auxin biosynthesis is also important for sustaining and maintaining nodule function. In addition, the specific pattern of promoter activity of *GmYUC2a* might provide a reasonable explanation for why overexpression of *GmYUC2a* leads to a significant delay in nodule development.

Phytohormones, such as auxin and cytokinin, have long been shown to play a role in legume nodulation (Libbenga *et al.*, 1973; Tirichine *et al.*, 2006, 2007; Wasson *et al.*, 2006; Murray *et al.*, 2007; Plet *et al.*, 2011; Suzaki *et al.*, 2012; Turner *et al.*, 2013; Wang *et al.*, 2015; Cai *et al.*, 2017). With regards to auxin, recent studies have reported that genes involved in auxin signaling and transport are correlated with soybean nodule development (Turner *et al.*, 2013; Wang *et al.*, 2015; Cai *et al.*, 2017). Changes in auxin transport have been proposed to determine auxin accumulation in nodule primordia (Suzaki *et al.*, 2013). Concomitantly, specific patterns of auxin accumulation could be associated with auxin biosynthesis induced by rhizobial infection to regulate nodule development

(Suzaki *et al.*, 2012, 2013). Moreover, auxin accumulation in root hairs and the expression of *LjTar1*, which is involved in auxin biosynthesis, are up-regulated in response to the Nod factor signaling pathway (Nadzieja *et al.*, 2018).

NIN has a well-established role in rhizobial infection and nodule organogenesis through the LHK1-dependent cytokinin signaling pathway (Schauser *et al.*, 1999; Marsh *et al.*, 2007; Tirichine *et al.*, 2007; Murray *et al.*, 2007; Suzaki *et al.*, 2013). Up-regulation of *LjTar1* appeared in the *Ljnin-2* mutant in response to Nod factor inoculation (Nadzieja *et al.*, 2018). However, in soybean, we found that *GmNIN* showed a stable increase in expression in response to exogenous auxin treatment and the overexpression of *GmYUC2a*, which is involved in auxin biosynthesis (Fig. 6A–C). This indicates that *GmNIN* expression could potentially be regulated by auxin production. Up-regulation of *GmNIN* in response to an increased auxin content may lead to the dedifferentiation and proliferation of cortical cells required for nodule organogenesis (Suzaki *et al.*, 2012). Constitutive expression of *NIN* also results in the formation of spontaneous nodule structures by regulating localized auxin responses in *L. japonicus* (Suzaki *et al.*, 2012; Soyano *et al.*, 2013). Curiously, the increased abundance of *GmNIN* transcripts induced by *GmYUC2a* overexpression failed to induce spontaneous nodulation in soybean. Clearly more research is required to elucidate how auxin and other hormones, such as cytokinin and gibberellin, interact to modulate nodule development.

A role for auxin in legume nodulation has long been proposed, but the source of auxin has been elusive. Here, we provide genetic evidence for the auxin biosynthesis gene *GmYUC2a* as having an important contribution to local auxin production that regulates both rhizobial infection and nodule organogenesis in soybean. Rhizobia induce the expression of *GmYUC* genes (mainly *GmYUC2a*) in the root to activate auxin biosynthesis, resulting in a local increase in auxin and the onset of auxin signaling. A possible model may be that auxin signaling is integrated with other hormone signaling pathways to reactivate the cell cycle in root hairs and the cortex for infection thread formation, nodule primordium initiation, and nodule development. These findings enhance our understanding of auxin regulation of nodule development in soybean, and provide novel insights into the important legume symbiosis.

Supplementary data

Supplementary data are available at JXB online.

Fig. S1. Phylogenetic relationship of YUC family proteins and the structures of their associated genes.

Fig. S2. *YUCCA* family gene expression in soybean.

Fig. S3. *GmYUC2a* transcript levels and root hair density were increased in *35S::GmYUC2a* transgenic roots.

Fig. S4. *GmYUC2a* overexpression inhibited primary root growth in Arabidopsis.

Fig. S5. Hairy root development was affected by 2,4-D treatment.

Fig. S6. *GmYUC2a* overexpression did not lead to root hair deformation without rhizobial inoculation.

Fig. S7. *GmYUC2a* expression in EV or 35S::*GmYUC2a* transgenic roots of soybean.

Fig. S8. *GmYUC2a* transcription in transgenic roots having EV or 35S::*GmYUC2a*.

Fig. S9. *GmYUC2a* silencing did not affect nodule development.

Table S1. The *YUC* family genes in *Glycine max*.

Table S2. The expression data of *GmYUC* family genes in different tissues of soybean (RPKM normalized data).

Table S3. Primers used in this study.

Dataset S1. Predicted amino acid sequence of AtYUCs, GmYUCs, and MedtrYUCs.

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