GmbZIP1 negatively regulates ABA-induced inhibition of nodulation by targeting GmENOD40–1 in soybean

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Abstract

Background: Abscisic acid (ABA) plays an important role in plant growth and adaptation through the ABA signaling pathway. The ABA-responsive element binding (AREB/ABF) family transcriptional factors are central regulators that integrate ABA signaling with various signaling pathways. It has long been known that ABA inhibits rhizobial infection and nodule formation in legumes, but the underlying molecular mechanisms remain elusive.

Results: Here, we show that nodulation is very sensitive to ABA and exogenous ABA dramatically inhibits rhizobial infection and nodule formation in soybean. In addition, we proved that GmbZIP1, an AREB/ABF transcription factor, is a major regulator in both nodulation and plant response to ABA in soybean. GmbZIP1 was specifically expressed during nodule formation and development. Overexpression of GmbZIP1 resulted in reduced rhizobial infection and decreased nodule number. Furthermore, GmbZIP1 is responsive to ABA, and ectopic overexpression of GmbZIP1 increased sensitivity of Arabidopsis plants to ABA during seed germination and postgerminative growth, and conferred enhanced drought tolerance of plants. Remarkably, we found that GmbZIP1 directly binds to the promoter of GmENOD40–1, a marker gene for nodule formation, to repress its expression.

Conclusion: Our results identified GmbZIP1 as a node regulator that integrates ABA signaling with nodulation signaling to negatively regulate nodule formation.

Keywords: ABA, Nodulation, GmbZIP1, GmENOD40–1, Soybean

Background

Root nodules are specialized root organ in leguminous plants developed under low nitrate conditions, where legumes and nitrogen-fixing bacteria (rhizobia) form a symbiotic relationship. In symbiotic nodules, legume plants provide bacteria with carbohydrates and energy for its growth and survival, conversely bacteria supply the plants with NH3 converted from the atmospheric N2 to meet the high nitrogen demand of legume plants [1–3].

The process of nodulation is initiated by a reciprocal and specific crosstalk between legumes and rhizobia. Under low nitrogen conditions, legume roots secrete flavonoid molecules into rhizosphere, which induce compatible rhizobia to synthesize and release signaling molecules called Nod factors (NFs) [4–6]. NFs are perceived by Lysine motif receptor-like kinases (LysM) receptors (NFRs) located at plasma membrane of the epidermal cells of roots and root hairs. These NF receptors include NF Perception (NFP) in M. truncatula, NF Receptors 1 and 5 (NFR1/5) in L. japonicus, SYM10/
SYM2 in *P. sativum* (pea) and NFR1α/β and NFR5α/β in *G. max* (soybean) [7–11]. The perception of NFs by these LysM receptors triggers a signaling cascade (the nodulation signaling) and a variety of cellular events to activate rhizobial infection and nodule formation. The key events following the NF perception are calcium spiking in the cytoplasm of infected root hair cells and transducing of the calcium signal to the nucleus by Does Not Make Infections 1 and 2 (DMI1 and DMI2) and Nuclear-localized cyclic nucleotide-gated channels (CNGC15) [12–14]. Calcium oscillations in the nucleus is decoded by Calcium and Calmodulin-dependent Kinase (CCaMK in Lotus and DMI3 in Medicago) that form complex with CYCLOPS to activate a set of transcriptional factors [15, 16]. These transcriptional factors include two GRAS domain transcriptional regulators [17–21], including Nodulation Signaling Pathway1 (NSP1) and NSP2, which can form a complex to directly activate expression of *Nodule Inception* (*NIN*), *Ethylene Response Factor Required for Nodulation* (*ERN1*) and *Early Nodulin 11* (*ENOD11*) [22, 23].

Phytohormones play a central role in symbiotic nodulation. Among them, cytokinin (CK) and auxin positively regulate symbiotic nodulation [24–29], while other hormones, such as jasmonate (JA), salicylic acid (SA), gibberellic acid, ethylene and ABA negatively regulate both rhizobial infection and nodule organogenesis [30]. Although ABA is well-known for its anti-stress effects in plants [31], researchers have also identified a negative role of ABA in nodulation of legumes. Exogenous application of ABA strongly reduced nodule number, while the treatment of Abamine, a specific ABA biosynthesis inhibitor increased nodule number [32–36]. ABA inhibited NF-induced calcium spiking in *M. truncatula*, suggesting that ABA affects nodulation at very early stage [35]. In *L. japonicus*, ABA treatment dramatically decreased the number of curled root hairs and the infection threads [33]. Consistent with these results, the induction of *nodulin* genes by NFs or rhizobia, such as *Rhizobium-induced Peroxidase* (*RIP*) and *ENOD11*, was abolished by ABA treatment [35]. Further genetic evidence that blocking the ABA signaling by overexpression of the Arabidopsis dominant negative allele *abi1–1* increased *nodulin* genes expression and subsequent nodule number demonstrates an inhibitory role of ABA in nodulation [35]. It appears that ABA suppressed the cytokinin-activated expression of a nodulation gene *ENOD40* that is involved in nodule formation in root cortex [35], although the detailed mechanisms by which ABA inhibits nodule and cross-talks to cytokinin are still to be elucidated.

ABA exerts its function through the canonical ABA signaling, which is a dynamic process that is initiated by the binding of ABA to the ABA receptors (the PYR1/PYLs/RCAR protein family), and involved the rapid transmission of the signal by group A protein type 2C phosphatases (PP2Cs) and sucrose nonfermenting-1-related protein kinase class 2 (SnRK2s) to the nucleus followed by ultimate activation of transcription factors resulting in altered expression of target genes [37–42]. ABA-responsive element (ABRE) binding protein/ABRE binding factors (AREB/ABF) transcription factors are responsible for the expression of most of the target genes, which are also known as ABRE/ABF regulon. In plants, these AREB/ABF transcription factors are basic region/leucine zipper (bZIP) class family proteins and can bind to the ABRE cis element in the promoters of ABA-responsive genes [43–45]. As a result, ABA can help to regulate plant response to abiotic stress [43–45]. Thus, the AREB/ABF transcription factors play a crucial role in controlling the activity of genes that regulate various aspects of plant response.

The inhibitory effect of ABA on soybean nodulation has been reported [32, 46–48]. It has been shown that ABA treatment markedly reduced production of isoflavonoid compounds, such as daidzein, genistein and coumestrol [32], thereby leading reduced rhizobial infection. However, no significant progress has been made in how ABA affects soybean nodulation since then. Based on the current evidence, soybean has the canonical ABA signaling, which consists of PYR1/PYLs receptors for ABA, PP2Cs, SnRKs and AREB/ABF transcription factors. For GmbZIPs, there are 160 GmbZIP genes identified in soybean genome, and few of them (e.g. *GmbZIP1* and *GmbZIP2*) has been functionally analyzed for their role in ABA response and stress tolerance [49, 50]. However, the role of the AREB/ABF transcription factors and the molecular mechanism by which ABA interplays with nodulation signaling remain completely unknown. In this study, we analyzed the effects of ABA on soybean growth and nodulation. We identified GmbZIP1, a soybean homolog of Arabidopsis AREB1, as a positive regulator of ABA response, but a negative regulator of soybean nodulation. *GmbZIP1* was induced by ABA and ectopic expression of *GmbZIP1* in Arabidopsis enhanced ABA sensitivity and plant tolerance to drought. Importantly, *GmbZIP1* was highly expressed in nodule, and overexpression of *GmbZIP1* resulted in reduced nodulation. Furthermore, we found that *GmbZIP1* directly bound to and repressed the expression of *GmENOD40–1*. These findings reveal for the first time the interplay between ABA and nodulation signal transduction pathway that fine tunes rhizobial infection and nodule formation in soybean.

**Results**

**ABA inhibits nodulation in soybean**

To assess the role of ABA during symbiotic nodulation in soybean, we first analyzed ABA effects on plant
growth and nodule formation. Six day-old soybean seedlings germinated on agar media were transferred onto Jensen’s plates [51] supplemented with 0, 0.5, 1 or 10 μM ABA and inoculated the plants with B. diazoefficiens USDA110. Eighteen days after ABA treatment and rhizobial inoculation, plant height, lateral root number and the number of root nodules were evaluated. As shown in Fig. 1a and b, shoot growth of the soybean seedlings was sensitive to ABA. Shoot growth was promoted by low concentration (0.5 μM) of ABA but was inhibited by high concentration (10 μM) of ABA. In comparison, lateral root formation of soybean seedlings showed different pattern in the conditions of ABA treatment (Fig. 1c and d). Lateral root formation of the plants treated with low concentrations (0.5 and 1 μM) of ABA was comparable to that of the control plants, however, lateral roots were significantly increased when treated with high concentration (10 μM) of ABA (Fig. 1c and d). In sharp contrast, nodulation was quite sensitive to ABA in soybean and nodule number strongly diminished with increased concentrations of ABA (Fig. 1c and e). The nodule number per plant under 0.5 μM ABA treatment was reduced to half of the control plants, and the nodule number was nearly completely abolished in the presence of 10 μM ABA. These results indicate that root nodule is the most sensitive organ of soybean to ABA during growth and development, and ABA plays a negative regulatory role in soybean nodulation.

Fig. 1 Exogenous ABA treatment reduces nodule number in soybean. a The phenotype of the aerial part of soybean treated with 0, 0.5, 1 or 10 μM ABA; Bar=7 cm; b Comparison of plant height of soybean with different concentration of ABA; c The phenotype of root and nodule of soybean with different concentration of ABA; d Comparison of primary lateral roots number of soybean treated with different concentration of ABA; e Comparison of root nodule number of soybean treated with different concentration of ABA. Bar = 7 cm. Values are means ± SD. ***: P < 0.001 and ns, P > 0.05.
ABA inhibits Rhizobial infection and NF signaling

The nodule formation begins with the reciprocal recognition between legume plant and rhizobia, which leads to the formation of infection foci [1]. To test if ABA affects rhizobial infection, we analyzed the number of infection foci with or without ABA treatment. In the presence of 10 μM ABA, the number of infection foci per plant was dramatically reduced compared with that of plant without ABA treatment (Fig. 2a and b), suggesting that ABA inhibits the rhizobial infection in soybean.

The reciprocal recognition of legume plant and rhizobia activates downstream signaling events, leading to rhizobia-specific infection and nodule formation [1]. To test whether ABA affects rhizobial infection by interrupting nodulation signaling, we analyzed the transcript levels of the nodulation marker gene GmENOD40–1 with or without ABA treatment. In the presence of rhizobia but without ABA treatment, GmENOD40–1 expression was highly induced by rhizobia at 3 DAI, but with ABA treatment, the induction of GmENOD40–1 was significantly reduced (Fig. 2c). The results indicate that ABA negatively regulates early nodulation through affecting the NF signaling transduction pathway.

Phylogenetic and expression analysis of ARBE family genes in soybean

AREB/ABF transcription factors are the central regulators of ABA responses in plants [43–45]. We wondered if the AREB transcription factors are involved in ABA-mediated suppression of soybean nodulation. AREB family genes encode the group A bZIP transcription factors and there are 28 group A members in soybean genome [49]. To search for the AREB transcriptional factors that involved in nodule organogenesis, we analyzed the evolutionary relationships between soybean and Arabidopsis. Similar to Arabidopsis [52], the 28 group A members in soybean can be classified in four distinct subgroups. Among them, GmbZIP11/GmbZIP1 [50] (thereafter GmbZIP1), GmbZIP29, GmbZIP44 and GmbZIP50 are classified into the same clade with AtABF1-AtABF4 (Fig. 3a), which function in the ABA-mediated abiotic stresses response [43–45], while GmbZIP61, GmbZIP84, GmbZIP91, GmbZIP96 and GmbZIP129 are grouped in the same branch as Arabidopsis ABI5 (Fig. 3a), which regulates ABA-mediated seed maturation and seed germination [53]. The result suggests potential function of these GmZIP transcription factors in ABA response in soybean.
To find out the candidate GmAREB genes associated with nodulation, we analyzed the expression levels of GmAREB genes in different tissues and organs using RNA sequencing (RNA-Seq) data obtained from SoyBase [54] (http://soybase.org/). Among these GmAREB genes, GmbZIP129 was nearly undetectable in all the tissues, four of them including GmbZIP61, GmbZIP84, GmbZIP91, GmbZIP96 were predominantly expressed in seed, which are similar to their homologous gene ABI5 in Arabidopsis [55]; and four genes including GmbZIP1, GmbZIP29, GmbZIP44 and GmbZIP50 were mainly expressed in flower and vegetative tissues including root hair and mature nodules (Fig. 3b). These data suggest that GmbZIP1, GmbZIP29, GmbZIP44 and GmbZIP50 may be involved in ABA-mediated nodule development in soybean.

**GmbZIP1 is predominantly expressed during nodule formation and nodule development**

To identify the GmAREB gene(s) that specifically function(s) in soybean nodulation, we performed qPCR to validate the expression patterns of GmbZIP1, GmbZIP29, GmbZIP44 and GmbZIP50 in soybean. Indeed, these four genes were differentially expressed in the tested tissues/organs including mature nodule at 28 DAI (Fig. 4a-d), suggesting diverse roles of these four GmAREB genes during soybean growth and development. Among them, GmbZIP44 and GmbZIP50 were expressed at the highest levels in root but lower in leaf, stem and nodule (Fig. 4c and d), while GmbZIP1 and GmbZIP29 were expressed in all the tissues with the highest levels in nodule (Fig. 4a and b). Notably, GmbZIP1 was predominantly expressed in nodule with very low or no expression in other organs, and its expression level in mature nodule was much higher than other GmbZIP genes (Fig. 4a). These results suggest that GmbZIP1 may play a major and specific role in soybean nodule development.

To further examine the expression pattern of GmbZIP1 during nodule formation and nodule development, we performed a histochemical analysis to test the promoter activity of GmbZIP1 using the GUS (β-glucuronidase) gene as a reporter. To do this, the 3 kb fragment upstream of ATG of GmbZIP1 was fused to the gusA gene and the resulting GmbZIP1pro:GUS construct was used for hairy root transformation and subsequent rhizobial nodulation. Histochemical analysis of GmbZIP1pro:GUS transgenic hairy roots was conducted at the specified stages of nodulation. High GUS activity was observed in nodule primordium and GUS activity was strongest in the cells at the base of nodule primordium (Fig. 4e). A high level of GUS activity was mainly

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**Fig. 3** Phylogenetic and expression analysis of soybean AREB family genes. **a** Phylogenetic analysis of the group A bZIP family members in soybean and Arabidopsis. The amino acid sequences of the group A bZIP family member were used for the alignment, and the phylogenetic neighbor-joining tree was constructed using MEGA5 phylogenetic analysis software. **b** Expression level analysis of AREB family gene members from public data (http://soybase.org/).
observed at the base of emerging nodule (Fig. 4f), and GUS activity remained high in young nodule and mature nodules (Fig. 4g and h). These observations indicate that $GmbZIP1$ expression is activated during nodule formation and nodule development.

$GmbZIP1$ is responsive to ABA and Positively regulates plant response to ABA

It has been previously shown that $GmbZIP1$ was induced by ABA in G. max L. cv. Tiefeng 8 and $GmbZIP1$ overexpression in Arabidopsis resulted in increased ABA sensitivity of plants [50]. To see if $GmbZIP1$ in Williams 82 is also ABA responsive, we analyzed the promoter of $GmbZIP1$ in 28 DAI. Seven-day-old seedlings were inoculated with $B. diazoefficiens$ strain USDA110. Expression pattern of $GmbZIP1$ in roots, stem, leaves, and nodules harvested at 28 DAI. Seven-day-old seedlings were inoculated with $B. diazoefficiens$ strain USDA110. Expression pattern of $GmbZIP1$ in roots, stem, leaves, and nodules harvested at 28 DAI. Seven-day-old seedlings were inoculated with $B. diazoefficiens$ strain USDA110. Expression pattern of $GmbZIP1$ in roots, stem, leaves, and nodules harvested at 28 DAI. Seven-day-old seedlings were inoculated with $B. diazoefficiens$ strain USDA110. Expression pattern of $GmbZIP1$ in roots, stem, leaves, and nodules harvested at 28 DAI. Seven-day-old seedlings were inoculated with $B. diazoefficiens$ strain USDA110. $GmZIP1$ was used as endogenous control. $GmBZIP1pro:GUS$ activity in nodule primordium at 3 DAI. Bar = 0.2 mm. $GmBZIP1pro:GUS$ activity in emerging nodule at 5 DAI, Bar = 0.2 mm. GUS activity in young nodule at 14 DAI, Bar = 1 mm; H GUS staining of $GmBZIP1$ in mature nodule at 28 DAI, Bar=5 mm.

To investigate whether $GmbZIP1$ in Williams 82 has the conserved function in plant ABA response, we compared the coding sequences of $GmZIP1$ genes between Tiefeng 8 and Williams 82, and found only six nucleotide difference between Tiefeng 8 and Williams 82 $GmbZIP1$s, which causes only four amino acid difference outside of the conserved domains in the corresponding proteins (Supplemental Fig. 2). To see whether the function of the $GmbZIP1$ from Williams 82 in ABA response remains unchanged, we also ectopically overexpressed $GmbZIP1-GFP$ under control of the CaMV 35S promoter in Arabidopsis. As shown in Fig. 5b, high intensity of GFP signal was observed in nucleus of $GmbZIP1-GFP$ transgenic root cells, suggesting that $GmbZIP1$ was successfully expressed in Arabidopsis ($GmbZIP1OE$) and $GmbZIP1$ is a nuclear protein. We then tested the ABA sensitivity of the Arabidopsis plants overexpressing $GmbZIP1$. In the absence of ABA, the rates of seed germination and cotyledon greening of $GmbZIP1OE$ plant were similar to that of the wild type (Fig. 5c and e). By contrast, in the presence of ABA, both seed germination and cotyledon greening rates of $GmbZIP1OE$ plant were significantly lower than that of wild type (Fig. 5d and f), suggesting that ectopic expression of $GmbZIP1$ resulted in increased ABA sensitivity of Arabidopsis plants. ABA is a key regulator of plant drought tolerance [56]. To see whether $GmbZIP1$ affects plant response to drought stress, we also evaluated drought tolerance of $GmbZIP1OE$ plants after withdrawing water and rehydration. Survival rate of $GmbZIP1OE$ plants under drought conditions was much higher than that of wild type...
(Supplemental Fig. 3), which is consistent with the previous result about a role of GmbZIP1 in drought tolerance [50]. Taken together, these results demonstrate that GmbZIP1 in Williams 82 is a positive regulator of plants in response to ABA response.

GmbZIP1 negatively regulates Rhizobial infection and nodule number

The next immediate question is whether GmbZIP1 functions in rhizobial infection and nodule organogenesis in soybean. To do this, we generated transgenic composite plants overexpressing GmbZIP1 under the control of the ubiquitin promoter (UBQ::GmbZIP1) (Fig. 6a), and examined the influence of GmbZIP1 overexpression on root hair deformation and nodule number at early and late stages of nodulation. As shown in the result (Fig. 6b and c), the number of the deformed root hairs in UBQ::GmbZIP1 hairy roots was enormously decreased compared with that in empty vector control at 7 DAI, suggesting a crucial role of GmbZIP1 in early stage of rhizobial infection. We then scored the nodule number at 14 DAI, and found that the transgenic hairy roots overexpressing GmbZIP1 produced fewer nodules than the empty vector control (Fig. 6d-f, Supplemental Fig. 4). These data reveal a negative role of GmbZIP1 in rhizobial infection and nodule development.

GmbZIP1 directly binds and represses expression of GmENOD40–1

To see whether GmbZIP1 inhibits rhizobial infection and nodule development through the nodulation signaling pathway, we analyzed the effect of GmbZIP1...
overexpression on the well-known nodulation marker gene *GmENOD40–1* using qPCR [57]. Intriguingly, the expression level of *GmENOD40–1* in the *UBQ::GmbZIP1* transgenic roots was substantially reduced compared to the vector control at 14 DAI (Fig. 7a). This result indicates that GmbZIP1 modulates soybean nodulation through the nodulation signaling pathway.

GmbZIP1 is a putative transcription factor and we speculated that GmbZIP1 may directly bind to the promoter of *GmENOD40–1* and repress its expression or downregulates *GmENOD40–1* by targeting the upstream positive regulators in the nodulation pathway. To this end, we first analyzed *cis* elements in the 3 kb *GmENOD40–1* promoter. Interestingly, there was one AREB *cis*-element in the promoter of *GmENOD40–1* (911 bp upstream of ATG) (Fig. 7b), suggesting a possible interaction between GmbZIP1 protein and the *GmENOD40–1* promoter. To prove the binding of GmbZIP1 to the *GmENOD40–1* promoter, we performed a DNA electrophoretic mobility shift assay (EMSA). For this, we expressed and purified the DNA binding domain of GmbZIP1 fused with a His tag in *Escherichia coli*. The result showed that the GmbZIP1-His fusion protein was able to bind to the DNA probes containing the AREB *cis*-element at the regions of the *GmENOD40–1* promoter (Fig. 7c, Supplemental Fig. 5). In a competition assay, the binding of GmbZIP1-His to the labeled DNA probe was reduced significantly by an excess amount of biotin-unlabeled DNA probe (Fig. 7c, Supplemental Fig. 5), suggesting that the interaction between GmbZIP1 and the *GmENOD40–1* promoter was specific. Collectively, these data suggest that GmBZIP1 regulates ABA-mediated nodulation inhibition by directly targeting *GmENOD40–1* and repressing its transcription.

**Discussion**

Symbiotic nodulation is a complex trait that is precisely regulated by endogenous cues and environmental conditions. Phytohormones play an integral role in overall nodulation. Auxin and cytokinin positively regulate nodulation, while others negatively regulate nodulation. Therefore, dynamic hormonal balance is crucial to optimal and successful interaction between rhizobia and plants and nodule formation [36]. It has been shown that ABA application inhibits both indeterminate and determinate nodules in legumes [33, 35, 46]. However, how ABA regulates nodulation, in particular how ABA signaling interplays with nodulation signaling remains elusive.
In this study, we showed that nodulation is the most sensitive process during plant growth and development; we identified GmbZIP1 as positive regulator of ABA response but negative regulator in soybean nodulation; and most importantly, we revealed that GmbZIP1 inhibits nodulation through directly repressing GmENOD40–1 expression.

ABA is a key regulator of plant growth and development [56]. In soybean, we found that exogenous ABA has diverse roles on shoot growth, lateral root development and nodulation. Low level of ABA stimulated shoot growth, while high level of ABA inhibited growth of shoot. In comparison, lateral roots were insensitive to ABA in lower ABA concentration, and high concentration of ABA promoted lateral root development. Interestingly, root nodule exhibited extremely sensitivity to ABA and ABA treatment reduced nodule number in a dose-dependent manner. Notably, nodulation inhibition mediated by ABA in soybean is specific, as the low concentration ABA (0.5 μM) was sufficient to inhibit nodulation but had no effect on shoot and root growth (Fig. 1e). Exogenous ABA can interrupt rhizobia-induced root hair deformation and nodule number in soybean, supporting the notion that during nodulation, ABA mainly exerts its negative regulatory role in early stage of nodulation including rhizobial infection and nodule formation as proposed before in M. truncula and L. japonicum [33, 35]. It is conceivable that the hypersensitivity of early nodulation to ABA is a conserved mechanism for attenuating nodulation signaling to maintain optimal nodule number in legumes.

Nodulation signaling pathway is responsible for rhizobial infection and nodule formation. It has been proposed that ABA inhibits rhizobial infection and nodule initiation through suppressing upstream signaling nodes in epidermis and activation of nodule formation genes, such as ENOD11 gene [35]. Consistent with this, our results showed that ABA exerts its inhibitory role through a similar mechanism in soybean. For example, the induction of GmENOD40–1 in soybean nodule formation was significantly reduced by ABA treatment (Fig. 2d). ABA regulates various biological processes mainly through ABA signaling. The previous results have identified Sensitivity to ABA 1 (STA-1) as a key ABA-mediated suppressor of the initiation of Nod factor signaling in Medicago [35], although the underlying molecular mechanism is not yet clear. However, the gene that mediates ABA-induced inhibition of nodule formation and its action remains completely unknown. Here, we provide several pieces of evidence to show that AREB transcription factor GmbZIP1 is a repressor of GmENOD40–1 to suppress nodule initiation induced by ABA.

AREB family proteins are key transcription factors in the ABA signaling pathway that regulate plant responses to ABA by targeting many genes related to plant growth and stress tolerance [44, 45]. Firstly, we found that among the ABA-related AREB/ABF genes in soybean, GmbZIP1 is specifically expressed in nodule primorida and nodule (Fig. 4). Secondly, we proved that GmbZIP1 is a positive regulator of plant ABA response. GmbZIP1 is induced by ABA and ectopic expression of GmbZIP1 increased ABA sensitivity of plant during early development and drought tolerance in Arabidopsis (Fig. 5, Supplemental Fig. 3). Thirdly, we demonstrated that GmbZIP1 negatively regulates soybean nodulation as overexpression of GmbZIP1 resulted in reduced number

![Fig. 7 GmbZIP1 binds and represses GmENOD40–1 expression. a qRT-PCR analysis of GmENOD40–1 expression in transgenic empty or UBQ::GmbZIP1 overexpression roots at 14 DAI. Values are mean ± SD, p< 0.001. b Schematic representation of GmENOD40–1 locus. The GmENOD40–1 promoter was shown as a line and the ACGT motif (ACGTGT) was shown as grey box. c EMSA for DNA binding domain of GmbZIP1 fused with a His tag to oligo-DNAs containing ACGTGT. Biotin-labeled probe was incubated with GmbZIP1-His. Competition for binding was performed with different concentration of unbiotin-labeled probe. The experiments were repeated for three times, and the representative results were shown.

In this study, we showed that nodulation is the most sensitive process during plant growth and development; we identified GmbZIP1 as positive regulator of ABA response but negative regulator in soybean nodulation; and most importantly, we revealed that GmbZIP1 inhibits nodulation through directly repressing GmENOD40–1 expression.
of root hair deformation and nodules (Fig. 6). Finally, we showed that GmbZIP1 can directly bind to the AREB element in the GmENOD40–1 promoter to repress its expression (Fig. 7). It is possible that rhizobia induces ABA to activate GmbZIP1 expression in cortex cells, which in turn represses GmENOD40–1 expression resulting in suppression of nodule formation. Since GmbZIP1 is expressed in other stages of nodulation and GmbZIP1 overexpression can also affect root hair deformation, we do not exclude the possibility that GmbZIP1 regulates ABA-mediated other processes during nodulation. Since GmbZIP1 is responsive and mediates plant response to abiotic stress [50], it is also possible that GmbZIP1 mediates nodulation under abiotic stress.

Conclusion

In summary, our data have identified a key component GmbZIP1 in ABA signaling of soybean that plays a negative role in soybean nodulation. Intriguingly, our findings reveal a novel mechanism by which GmbZIP1 mediates crosstalk between the ABA and NF signal transduction pathways during nodule initiation. Considering high level of conservation in ABA-mediated nodulation inhibition and bZIP1 transcription factor in legumes, our findings provide novel insights into elucidation of ABA regulation of symbiotic nodulation.

Methods

Plant and rhizobia growth, hairy root transformation, and B. diazoefficiens inoculation

Soybean [G. max (L.) Merrill cv. Williams 82] was used to clone and analyze the function of GmbZIP1. The seeds were obtained from Lab of Prof. Tianfu Han (Chinese Academy of Agricultural Sciences). Soybean seedlings were cultured in a growth room at 25–26 °C and 16 h/8 h light/dark conditions. For soybean hairy root transformation, healthy and uniform soybean seeds were planted in soil for 3 days in growth room. Germinating seedlings were used for hairy root transformation with Agrobacterium rhizogenes strain USDA110 (OD600 = 0.08) as described previously [58]. For nodulation phenotype assays, the putative transgenic plants were transplanted to pots containing vermiculite and grown for 1 week to allow rooting. They were then inoculated with 30 ml suspension of B. diazoefficiens USDA110 (OD600 = 0.1). The plants were cultured in growth room and the plates were wrapped with silver paper to keep root in dark. 18 days later, the nodule number, shoot and root phenotype were analyzed.

Curled root hair assays

In order to examine the early infection events, the root segments (4 cm) below the hypocotyl–root junctions were cut from the transformed hairy roots at 7 DAI. The root fragments were rinsed briefly in sterile PBS buffer and stained with 0.01% methylene blue for 15 min and then washed three times with deionized water as described previously [57]. The stained transgenic roots were observed with a Leica biological microscope. The root hairs with tight curls were counted and referred to as 'Infection foci'.

RNA extraction and quantitative PCR analysis

Total RNA was extracted from wild type or composite plants using Trizol reagent according to the manufacturer's protocol (Tiangen Biotech Co., Ltd., Beijing, China). The total RNA samples were treated with DNase I (Invitrogen) to remove contaminating genomic DNA and first-strand cDNA was synthesized using a FastQuant RT Kit (Tiangen Biotech). Quantitative polymerase chain reactions (qPCR) were performed using SuperReal PreMix Plus (SYBR Green; Tiangen Biotech). GmELF1B was used as the internal reference gene [57].

Vector construction

For the overexpression construct of GmbZIP1, the full length GmbZIP1 was cloned and inserted into the vector pLI1BtpCAMBIASA1301 using the restriction enzymes XbaI/BamHI. For the GmbZIP1 promoter: GUS reporter construct, the putative promoter region (2.0 kb) of GmbZIP1 was amplified from cv. Williams 82 genomic
EMSA
Electrophoretic Mobility Shift Assays (EMSAs) were performed using the Light Shift Chemiluminescent EMSA Kit (Pierce) according to the manufacturer’s protocol and as described by Wang et al. [57]. For the GmbZIP1-His construct, the coding sequence of the GmbZIP1 (901–1320 bp) was amplified and inserted into pET22b using NdeI/HindIII. The probe-binding activity of the protein was analyzed using oligonucleotides labeled with biotin at the 5’end (Sangon, China). After incubation at room temperature for 30 min, the protein-probe mixture was separated on a 6% polyacrylamide gel and transferred to a Biodyne B nylon membrane (Pall). Migration of the biotin-labeled probes was detected using streptavidin-horseradish peroxidase conjugates that bind to biotin and the chemiluminescent substrate according to the manufacturer’s protocol.

Statistical analysis
The data including gene expression and phenotypic analysis in this study were analyzed using GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA). The averages and Standard Deviations (SDs) of the data were calculated, and one-way ANOVA Student’s t test were performed to generate p values.

Supplementary Information
The online version contains supplementary material available at https://doi.org/10.1186/s12870-020-02810-9.

Abbreviations
ABA: Abscisic acid; ABF: ABRE binding factor; ABRE: ABA-responsive element; bZIP: Basic region/leucine zipper; CCaMK: Calmodulin-dependent Kinase; CK: Cytokinin; CNGC15: Nuclear-localized cyclic nucleotide-gated channels; DMI1: Does Not Make Infections 1; ENOD11: Early Nodulin 11; ERN1: Ethylene Response Factor Required for Nodulation1; JA: Jasmonate; NF: Nod factor; NFR: NF receptor; NIN: Nodule Inception; PP2C: Group A protein type 2C phosphatases; RIP: Rhizobium-induced Peroxidase; SA: Salicylic acid; SnRK2s: Sucrose nonfermenting-1-related protein kinase class 2.

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Authors' contributions
ZW and XL conceived the study and designed the experiments; SX, SS, XD, and Standard Deviations (SDs) of the data were calculated, and one-way ANOVA Student’s t test were performed to generate p values.

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Availability of data and materials
All data generated or analysed during this study are included in this published article and its supplementary information files. The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate
Not applicable.

Consent for publication
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Competing interests
The authors declare that they have no competing interests.

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