The NF-Y-PYR module integrates the abscisic acid signal pathway to regulate plant stress tolerance

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Summary

Drought and salt stresses impose major constraints on soybean production worldwide. However, improving agronomically valuable soybean traits under drought conditions can be challenging due to trait complexity and multiple factors that influence yield. Here, we identified a nuclear factor Y C subunit (NF-YC) family transcription factor member, GmNF-YC14, which formed a heterotrimer with GmNF-YA16 and GmNF-YB2 to activate the GmPYR1-mediated abscisic acid (ABA) signalling pathway to regulate stress tolerance in soybean. Notably, we found that CRISPR/Cas9-generated GmNF-YC14 knockout mutants were more sensitive to drought than wild-type soybean plants. Furthermore, field trials showed that overexpression of GmNF-YC14 or GmPYR1 could increase yield per plant, grain plumpness, and stem base circumference, thus indicating improved adaptation of soybean plants to drought conditions. Taken together, our findings expand the known functional scope of the NF-Y transcription factor functions and raise important questions about the integration of ABA signalling pathways in plants. Moreover, GmNF-YC14 and GmPYR1 have potential for application in the improvement of drought tolerance in soybean plants.

Introduction

Global climate change has exacerbated the damage caused by drought, temperature, and salt stresses in many grain-producing areas around the world and currently presents many challenges for crop production. Plants have evolved defence systems and sophisticated mechanisms that enable their adaptation to changing environments (Zhu, 2002). These systems include plant hormones and related signalling pathways that play important roles in regulating plant responses to environmental stresses (Zhu, 2002; Engelberth and Engelberth, 2009). Changes in the levels of plant hormones under stress conditions enable cells to sense stress signals and initiate an appropriate defence response. Among plant hormones, abscisic acid (ABA) performs several roles in mediating abiotic stress responses. When plants are exposed to abiotic stress, changes in ABA levels can result in the up-regulation of genes encoding various proteins necessary for stress resistance and maintenance of life cycle processes (Zhu, 2002). Therefore, the elucidation of molecular mechanisms that participate in plant stress resistance is critical for ameliorating the effects of environmental stresses on plant growth.

ABA signalling can effectively limit water loss through transpiration by regulating stomatal aperture and up-regulating of stress-responsive genes (Chandler, 1994; Zhu, 2016). The ABA pathway also has crosstalk with the jasmonic acid, salicylic acid, and ethylene signalling pathways (Ku et al., 2018). Genes which are typically up-regulated in response to ABA or drought are commonly down-regulated in jaz mutants, indicating the existence of crosstalk between the ABA and jasmonic acid signalling pathways (Seonghee et al., 2018). In Arabidopsis, the overexpression of ERF1 was shown to increase resistance to drought stress by up-regulating the expression of stress-responsive genes, accumulating ABA and proline, and closing stomata; these findings indicate the presence of interactions between the ABA and ethylene signalling pathways (Cheng et al., 2013). Other molecular studies have shown that transcription factors such as AP2/EREBP, WRKY, NAC, and MYB play crucial roles in mediating resistance to abiotic stresses by regulating upstream ABA signalling, which then activates a cascade of downstream genes (Chinnusamy et al., 2007). However, among these transcription factors, the relationship between the nuclear factor Y (NF-Y) transcription factor, ABA, and plant stress resistance remains unclear. Previous studies have shown that LEAFY COTYLEDON 1 (LEC1/NF-YB9) and LEC1-LIKE (L1L/NF-YC2) interact with bZIP67 to regulate ABA-related gene expression (Yamamoto et al., 2009), suggesting that NF-Y could regulate the ABA-associated signalling pathway.

NF-Y is highly conserved, ubiquitous transcription factor found in almost all eukaryotes. It consists of three subunits, including NF-YA, NF-YB, and NF-YC, which interact to form an integrated transcription factor structure (Bucher, 1990; McNabb et al., 1995;
Sinha et al., 1995); all three subunits are required for binding the CCAAT cis-element. NF-Y genes are also involved in the regulation of various plant developmental processes. Several genes encoding NF-Y subunits have been studied, although the plant NF-Y complex has not been completely characterized. LEC1/NF-YB9, the first reported plant NF-Y gene, is important for regulating embryo development and seed maturation, and loss-of-function mutants for this gene produce defective embryos (Meinke et al., 1994; West et al., 1994). Recently, NF-Y genes were found to regulate flowering time (Ben-Naim et al., 2006; Cai et al., 2007; Kumimoto, Zhang et al., 2010; Liang et al., 2012; Wenkel et al., 2006). Under long-day condition, NF-YC2 and NF-YC1 overexpression lines exhibit a significant early flowering phenotype, while nf-yc2 and nf-yc1-2 mutants exhibit significantly delayed flowering compared with wild-type (WT) plants (Hackenberg et al., 2012). These findings thus demonstrate the involvement of NF-YC genes in floral induction. NF-Y also plays important roles in facilitating plant responses to various environmental signals and hormones.

Here, in this study, we investigated the mechanism through which the NF-Y complex regulates transcription to control plant responses to drought and salt stresses. In particular, we show the regulatory mechanism by which NF-Y controls the ABA-associated signalling pathway and provide a summary of these interactions. Furthermore, using the soybean drought transcriptome database, we identified the GmNF-YC14 gene, which encodes a NF-YC family protein. We found that GmNF-YC14 expression was induced by drought, salt, and ABA treatments. Overexpression of soybean GmNF-YC14 in Arabidopsis enhanced sensitivity to ABA and improved the tolerance of transgenic Arabidopsis to drought and salt stresses. Further, RNA-seq of transgenic soybean and WT plants with subsequent functional analysis revealed a novel regulatory module in which GmNF-YC14 interacts with GmNF-YA16 and GmNF-YB2 to form a heterotrimer when the plant is exposed to drought and salt stresses. This heterotrimer complex can directly regulate a PYR/PYL family ABA receptor, resulting in the activation of ABA signal transduction, and hence, an enhanced response to stress.

**Results**

**Drought stress alters the expression of multiple soybean NF-YC genes**

To screen genes with altered expression in the soybean response to drought stress, we performed RNA-seq on plants treated with water-deficit or full irrigation conditions. Given their extensive functional impacts, we were particularly interested in the differential expression of transcription factor. Our results showed that genes from several families of transcription factor were differentially expressed in response to drought stress. Stress-induced changes in expression were particularly pronounced for genes encoding NF-Y family of transcription factors (Figure S1). Specifically, drought stress resulted in the increased expression of multiple NF-YC genes (Figure S1d).

To investigate NF-YC function in soybean tolerance to drought stress, we identified 25 soybean NF-YC family member genes by multiple sequence alignment and categorized these genes into four groups (Figure S2). Real-time quantitative PCR (qPCR) was used to measure their expression levels under drought, salt, and cold stress conditions, as well under activation by exogenous ABA (Figure S3). Among these NF-YC genes, GmNF-YC14 expression was differentially induced by ABA application as well as under drought and high salt conditions. When soybean plants were subjected to drought stress or ABA treatment, GmNF-YC14 expression increased, peaking at 2 and 12 h, respectively (Figure S2,S4a). Similarly, the expression of GmNF-YC14 was induced in response to salt stress and peaked at 30 min after stress induction, then declined after 24 h (Figure S3,S4a). We then selected GmNF-YC14 for further analysis to identify other genes that might participate in conferring abiotic stress tolerance.

**Overexpression of GmNF-YC14 confers drought and salt tolerances in Arabidopsis**

To better understand the functions of soybean GmNF-YC14, we generated transgenic Arabidopsis lines expressing this gene under the control of CaMV 35S promoter and selected three independent overexpression lines for GmNF-YC14 (GmNF-YC14-OE1, -OE2, and -OE3) phenotypic analysis in pot experiments under different stress conditions. Drought stress was induced in three-week-old Arabidopsis seedlings by exposure to water-deficit conditions for 1 week, followed by re-watering in a 3 day recovery period. As shown in Figure S4b, following the recovery period, more than 86.1% (average of three lines) of the GmNF-YC14-OE plants survived, compared with only 8.5% of WT plants (Figure S4c). The same phenotype was observed after 5 days of 250 mM NaCl treatment in pot experiments, with more than 72.8% of the GmNF-YC14-OE plants surviving, compared with 16.5% survival in WT plants (Figure S4d). We also found that under drought and salt conditions, chlorophyll content also increased by 32.2% and 54.1%, respectively, in the GmNF-YC14-OE plants compared with WT plants (Figure S4e,f). These data indicated that overexpression of GmNF-YC14 could enhance tolerance to drought and salt conditions in Arabidopsis.

**Effect of overexpression of GmNF-YC14 in Arabidopsis on ABA sensitivity**

To explore the connection between high expression of GmNF-YC14, drought tolerance, and ABA signalling, we next monitored ABA-mediated stomatal closure in GmNF-YC14-OE and WT plants. To this end, we placed leaves of 3-week-old Arabidopsis plants into stomatal-opening solution to induce complete opening, then exposed these leaves to a range of ABA concentrations for 2.5 h. The mean stomatal aperture — calculated as the ratio of stomata width to length — of both WT and GmNF-YC14-OE leaves decreased with increasing concentrations of ABA (Figure S4g). However, guard cells in the GmNF-YC14-OE lines exhibited greater sensitivity to ABA-induced stomatal closure compared with those of the WT leaves (Figure S4h). Moreover, stomata of the GmNF-YC14-OE leaves closed more rapidly than WT stomata following treatment with exogenous ABA. Comparison of the rates of water loss between detached leaves of WT plants and GmNF-YC14-OE lines during dehydration revealed that the GmNF-YC14-OE plant leaves exhibited lower rates of water loss (Figure S4i), which was consistent with the rapid stomatal closure observed in GmNF-YC14-OE plants following ABA treatment. These results suggested that detached leaves from the transgenic lines had a greater ability to withstand dehydration than leaves taken from WT plants.

**Tolerance to drought and salt is decreased in CRISPR/ Cas9-induced Gmnf-ycc14 soybean mutants and increased in GmNF-YC14-overexpressing soybeans**

To further analyse the function of GmNF-YC14 in soybean stress responses, we generated two independent GmNF-YC14-
overexpressing soybean lines (named GmNF-YC14-OE1 and -OE2) and two independent CRISPR/Cas9-mediated loss-of-function mutants (named GmNF-YC14-KO1 and -KO2). We then identified T3 homozygous GmNF-YC14-OE and GmNF-yfc14-KO mutant soybean plants by DNA sequencing and qPCR screens. DNA sequencing confirmed that two or five bases (AC or CGTA) upstream of the PAM site were lost in two GmNF-yfc14-KO mutants, respectively, which resulted in amino acid conversions (Figures 1a,b, Figure S5a). Additionally, qPCR confirmed that GmNF-YC14 transcript levels were higher in GmNF-YC14-OE lines than that in the WT plants (Figure S5b,c). Subsequently, the GmNF-YC14-OE and GmNF-yfc14-KO soybean plants were subjected to treatment with drought or 300 mM NaCl. Under drought conditions, Gmnf-yfc14-KO plants began wilting on day 5 and were flaccid by day 10 (Figure 1c). Following the three-day recovery period, 35% WT recovered, whereas 5% of mutant plants were severely dehydrated and could not be revived (Figure 1d). Following 3 days of NaCl treatment, both Gmnf-yfc14-KO and WT plants began to wilt and were seriously affected by day 5 (Figure 1e and 1f). By contrast, relatively fewer leaves of the GmNF-YC14-OE lines began to wilt only after 5 days of NaCl treatment, and their green coloration persisted longer than that in the WT and GmNF-yfc14-KO plants (Figure 1f). Measurements of biomass (fresh weight of aerial parts, fresh weight of the roots, and chlorophyll content) for Gmnf-yfc14-KO, WT, and GmNF-YC14-OE plants revealed that GmNF-YC14-OE seedlings had the highest biomass under both drought and salt stress conditions (Figure 1g–l and Figure S6).

We next evaluated tolerance to drought and salt stresses in GmNF-yfc14-KO, WT, and GmNF-YC14-OE plants at the flowering stage and found that the results were consistent with those obtained at the seedling stage (Figure 2a). Physiological and biochemical index analyses revealed that GmNF-YC14-OE plants had higher proline and lower malondialdehyde (MDA) contents compared with WT plants under drought and 500 mM NaCl treatments. In contrast, Gmnf-yfc14-KO plants exhibited lower proline and higher MDA content compared with WT plants (Figure 2b–e).

We also assessed drought resistance and plant agronomic traits in the seed maturation stages after flowering. Under drought stress, the GmNF-YC14-OE plants exhibited stronger phenotypes for several agronomic traits than those of WT plants (Figure 2f–k), such as greater circumference at the stem base, higher grain number per plant, and higher grain weight per plant. By contrast, mutant plants exhibited relatively inferior phenotypes for these traits compared with the WT plants (Figure 2). Seed traits were also analysed, and the seeds were divided into four groups based on seed plumpness and weight, with Group I seeds having the highest plumpness and weight and Group IV having the lowest scores for these traits (Figure 2l and Table S1). We observed that GmNF-YC14-OE plants produced a larger number of Group I seeds and fewer Group IV seeds compared with WT under drought conditions (Figure 2m), Gmnf-yfc14-KO mutant plants exhibited the opposite trend, producing fewer Group I seeds and more Group IV seeds than WT. These findings further supported the hypothesis that the GmNF-YC14-OE plants were more resistant to drought than either mutant or WT plants.

Interactions between GmNF-YC14, GmNF-YB2, and GmNF-YA16

Protein–protein interactions are essential for many biological functions. To isolate the proteins interaction partners of GmNF-YC14, we conducted yeast two-hybrid assays using GmNF-YC14 as the bait. In the initial screen, GmNF-YA16 appeared to interact with GmNF-YC14. Considering that NF-Y reportedly functions as a heterotrimeric complex, we next focussed on identifying the NF-Y transcription factor B subunit protein. To this end, we generated the GmNF-YC14-pBridge-GmNF-YA16 plasmid construct to detect candidate interaction partners by yeast three-hybrid assay. In these experiments, GmNF-YA16 expression was driven by a conditional methionine (Met) promoter (pMSISU) such that its expression was activated in the absence of Met and repressed in the presence of Met. The plasmids (GmNF-YC14-pBridge-GmNF-YA16 and cDNA library) were then co-transformed into the yeast AH109 strain to detect putative protein–protein interactions between the NF-Y two-subunit construct and the unidentified component of the NF-Y transcription factor complex. This screen identified GmNF-YB2 as the strongest candidate for direct interactions.

We then used yeast two-hybrid and three-hybrid systems to investigate whether GmNF-YC14 interacted with GmNF-YA16 and GmNF-YB2, individually or together. Yeast two-hybrid assays confirmed that GmNF-YB2 likely interacted with GmNF-YC14 (Figure 3a). In the yeast three-hybrid assay, the two plasmids carrying GmNF-YC14-pBridge-GmNF-YB2 and GmNF-YA16-AD, respectively, were co-transformed into AH109 strains and the colonies were screened on drop-out media with or without Met. The co-transformed yeast cells grew normally on defective medium with Met (SD/Trp-/His-/Ade-), indicating that GmNF-YB2 expression was not induced. These results showed that GmNF-YC14 could interact with GmNF-YA16 (Figure 3b). Additionally, the co-transformed yeast cells grew normally on defective medium lacking Met (SD/Trp-/Leu-/His-/Ade-/Met-), and that the expression of GmNF-YB2 was induced. These findings indicated that GmNF-YC14 and GmNF-YB2 could both potentially interact with GmNF-YA16 (Figure 3c).

To verify these results, we also performed luciferase complementary imaging (LCI) and bimolecular fluorescence complementation (BiFC) assays. The LCI assay revealed LUC signals in the GmNF-YB2-cLUC and GmNF-YC14-nLUC co-injected areas of a tobacco leaf (Figure 3d). The same results were observed with GmNF-YB2-nLUC and GmNF-YA16-cLUC, as well as with GmNF-YC14-nLUC and GmNF-YA16-cLUC constructs (Figure 3e,f), thus confirming that GmNF-YB2 could interact with GmNF-YC14, GmNF-YB2 could interact with GmNF-YA16, and GmNF-YC14 could interact with GmNF-YA16. Similarly, the BiFC assays revealed nuclear YFP signals only in the regions of tobacco leaves where GmNF-YA16-nYFP and GmNF-YB2-cYFP; and GmNF-YC14-nYFP; and GmNF-YB2-cLUC; and GmNF-YA16-cLUC were co-injected (Figure S7a). Collectively, these results suggested the presence of mutual interactions between the GmNF-YB2, GmNF-YC14, and GmNF-YA16 proteins.

Next, we investigated the interactions between GmNF-YB2, GmNF-YC14, and GmNF-YA16 using in vitro pull-down assays. To this end, we purified recombinant MBP-tagged GmNF-YB2 (GmNF-YB2-MBP), GST-tagged GmNF-YC14 (GmNF-YC14-GST), and His-tagged GmNF-YA16 (GmNF-YA16-His) or GmNF-YB2 (GmNF-YB2-His). The ability of GmNF-YC14-GST to pull-down GmNF-YB2-His, but not the His tag alone, and the inability GmNF-YB2-His to bind the GST tag alone, together indicated that GmNF-YC14 and GmNF-YB2 interacted in vitro (Figure 3g). Similarly, we found that GmNF-YB2 and GmNF-YC14 both
physically interacted with GmNF-YA16 in vitro (Figures 3h,i). These results demonstrate that GmNF-YC14, GmNF-YA16, and GmNF-YB2 could all interact with each other, thus leading us to hypothesize that these three proteins could form a heterotrimer in vivo, thereby giving rise to an integrated NF-Y transcription factor complex.

Molecular characteristics and function analysis of in GmNF-YA16 and GmNF-YB2 subunit proteins

Based on our findings that GmNF-YA16 interacted with GmNF-YC14 and GmNF-YB2 in vitro, we next sought to characterize the functions of these interaction partners. We therefore individually cloned GmNF-YC14, GmNF-YB2, and GmNF-YA16 into the pJIT16318 subcellular localization vector, which contains a CaMV 35S promoter and a C-terminal GFP to observe their subcellular localization. Approximately $4 \times 10^4$ mesophyll protoplasts were isolated from Arabidopsis seedlings and transfected with GmNF-YC14/GmNF-YB2/GmNF-YA16-GFP plasmids via polyethylene glycol (PEG)-mediated transformation. We observed that GFP-only control was diffused throughout the plasma membrane, nucleus, and cytosol, whereas GmNF-YC14 was localized in the nucleus and plasma membrane; GmNF-YB2
was localized in the plasma membrane, nucleus, and cytosol; and GmNF-YA16 was localized specifically in the nucleus (Figure S7b).

To explore the potential roles of GmNF-YA16 and GmNF-YB2 in soybean response to abiotic stress, soybean hairy root assays were conducted by Agrobacterium \textit{K599}-mediated generation of transgenic GmNF-YA16 and GmNF-YB2 hairy root composite plants (Figure S8a,b). Exposure of these plant to water-deficit or 200 mM NaCl revealed that overexpression of either GmNF-YA16 or GmNF-YB2 in soybean hairy roots enhanced the tolerance of transgenic composite plants to drought and salt stresses compared with that of the empty vector control plants (Figure 3j–k).

Figure 2 Stress resistance of different soybean plants at the flowering and adult stage. (a–e) Phenotypic analysis of the different plants at the flowering stage under conditions of drought and salt stresses (a); MDA and proline content under conditions of drought (b, c) and salt (d, e) stresses. Blue and orange bars indicate the average of the two Gmnf-yc14-KO lines and two GmNF-YC14-OE lines, respectively. (f) Phenotypic analysis of the plants at the adult stage under drought stress conditions. (g–k) Number of pods per plant (g); plant height (h); grain weight per plant (i); stem base circumference (j); and grain number per plant (k). (l, m) Phenotypic analysis of seeds under conditions of drought stress at the adult stage (l) and the relative proportions of different seed types (m).
Moreover, measurements of biochemical and physiological indexes indicated that the GmNF-YA16 and GmNF-YB2 transgenic soybean hairy roots contained lower levels of MDA and higher levels of proline compared with soybean hairy roots harbouring the empty vector (Figure 3l–o). These results suggested that both GmNF-YA16 and GmNF-YB2 could participate in the plant response to abiotic stress.

Altered gene expression in GmNF-YC14 transgenic plants
To identify potential regulatory targets of the NF-Y transcription factor complex, we performed Illumina sequencing to characterize patterns of differential expression between the WT and transgenic soybean plants induced by drought stress. Whole

Figure 3 Interaction between candidate proteins and GmNF-YC14. (a–c) Interaction analysis of GmNF-YA16, GmNF-YC14, and GmNF-YB2 proteins by yeast two-hybrid (a) and three-hybrid (b, c) assays. (d–f) Luciferase complementation assay to verify the interaction. YA16, GmNF-YA16; YB2, GmNF-YB2; YC14, GmNF-YC14. (g–i) GST-pulldown assay of the three proteins. (j, k) Stress tolerance analysis of GmNF-YA16 and GmNF-YB2 candidate protein genes. (l–o) Physiological and biochemical index analysis of GmNF-YA16- and GmNF-YB2-overexpressing soybean hairy root composite plants under drought and salt stress conditions.
transcriptome sequencing data identified several putative target genes with significantly higher expression in GmNF-YC14-OE plants compared with WT, which suggested their involvement in mediating soybean response to drought. Functional enrichment and KEGG pathway analysis of the transcriptome data indicated that genes related to ‘response to stimulus’ and ‘the hormone

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signal transduction pathway' were highly enriched under drought stress, with significantly higher expression GmNF-YC14-OE plants (Figures 4a–b). Among these genes, we noted the up-regulated expression of a PYR/PYL family ABA receptor, GmPYR1, as well as eight stress response-related genes (GmABF1, GmABF2, GmABF3, GmABF4, GmMYB84, GmNAC4, GmDHN15, and GmRD22) in GmNF-YC14-OE plants compared with that in WT (Table S2, S3). To validate the RNA-seq data, we performed qPCR analysis of these nine genes and found that the trends in their expression generally corresponded to those in the RNA-seq data (Figure 4c). To further investigate the relationships between GmNF-YC14 and the functional genes identified in our RNA-seq data, we analysed the promoter sequences of GmABF1, GmABF2, GmABF3, GmABF4, GmMYB84, GmNAC4, GmDHN15, and GmRD22 and found that the promoters of all genes except for GmABF1 harboured CCAAT cis-acting elements. Thus, these seven promoters were isolated from the soybean genome using specific primers and cloned into the pGreenII0800 vector to determine the effects of GmNF-YC14 on their activation in dual-luciferase reporter assays. The results showed that all seven promoters expressed potent LUC activity upon co-transfection with GmNF-YC14 (Figure 4d–j), indicating that GmNF-YC14 could activate the transcription of these genes.

GmPYR1 can be regulated by the NF-Y transcription factor

In light of our above findings, we next explored the potential interactions between NF-Y complex proteins and GmPYR1, a key ABA receptor involved in the ABA signal transduction pathway. We therefore used qPCR to measure GmPYR1 transcriptional expression in GmNF-YA16- and GmNF-YB2-overexpressing soybean hairy roots and found that GmPYR1 expression was enhanced by the overexpression of GmNF-YA16 or GmNF-YB2 in soybean hairy roots (Figure 5a,b), which indicated that GmNF-YA16 and GmNF-YB2 could regulate GmPYR1 expression. Careful sequence analysis of the GmPYR1 promoter region revealed the presence of three CCAAT motif cis-acting elements (Figure 5c). To evaluate interactions between the promoter and NF-Y transcription factor proteins, we next cloned the full GmPYR1 promoter region into the pGreenII0800 vector for subsequent LUC activity and dual-luciferase reporter assays. These experiments showed that the GmPYR1 gene promoter strongly induced LUC activity in the presence of GmNF-YA16, GmNF-YB2, and GmNF-YC14 proteins (Figure 5d–f), thus suggesting that all three NF-Y subunits could apparently interact with the GmPYR1 promoter.

To verify these results, the three proteins were expressed with different fusion tags in E. coli, purified in vitro, and used for electrophoretic mobility shift assays (EMSA) with a biotin-labelled probe synthesized using the GmPYR1 promoter sequence (Figure 5g). The GmNF-YA16, GmNF-YB2, and GmNF-YC14 proteins formed a heterotrimeric complex in vitro and bound to the CCAAT cis-acting element (Figure 5g). Collectively, these results indicated that the NF-Y transcription factor could regulate the expression of the ABA receptor, GmPYR1.

GmPYR1 overexpression in soybean leads to enhanced stress tolerance

To elucidate the function of GmPYR1 in the soybean response to abiotic stress factors, we generated GmPYR1-overexpressing soybean plants and obtained two independent GmPYR1 transgenic lines (GmPYR1-OE1 and GmPYR1-OE2), which were confirmed by DNA sequencing and qPCR screening (Figure S8c–d). Phenotypic analysis of stress tolerance in the seedling and adult stages showed that the GmPYR1-OE plants exhibited higher tolerance to both drought and high salt conditions at the seedling stage than that of WT seedlings (Figure 5h–i). Moreover, the biomass of GmPYR1-OE plants was higher than that of the WT plants under these stress conditions (Figure 5j–m). We also noted that adult stage abiotic stress traits of GmPYR1-OE plants were more favourable under drought conditions than those of the WT plants (Figure 6); specifically, GmPYR1-OE plants displayed a greater circumference at the stem base, higher grain number per plant, and higher grain weight per plant (Figure 6b–f). Finally, seed traits were analysed as described above (Figure 6g). The results indicated that GmPYR1-OE plants produced a higher number of Group I seeds and a lower number of Group IV seeds than WT under drought conditions (Figure 6h). Overall, these findings indicated that GmPYR1-OE soybean plants exhibited an enhanced drought tolerance phenotype compared with that of WT plants.

ABRE-binding transcription factors activate the expression of abiotic stress-related genes

Drought can induce ABA accumulation in plants, and the PYL/PYL-ABA receptor protein has been shown to bind ABA to increase the activity of the ABA-responsive element (ABRE)-binding transcription factors, consequently activating the expression of stress-responsive genes (Zhao, 2016). Thus, ABRE-binding transcription factors (ABFs) are known to regulate the ABA pathway. In order to study the functions of ABFs in the soybean response to high salt or drought conditions, we used Agrobacterium K599-mediated transformation to generate a soybean hairy root line, each overexpressing one of four ABFs that showed high differential expression under drought stress in RNA-seq data from the GmNF-YC14-OE lines. This experiment showed that overexpression of these ABFs enhanced tolerance to both drought and salt stresses in the transgenic composite plants (Figure S9a,b).

Stress-responsive genes, such as NAC and MYB transcription factors or dehydrin, contribute essential regulatory functions to mediate plant stress resistance, and their expression can be induced via ABA signalling (Quach et al., 2014; Wang et al., 2017). Therefore, to clarify potential relationships between the two significantly up-regulated genes identified in the GmNF-YC14-OE de novo transcriptome data, we next used qPCR to examine the expression of GmRD22, GmNAC4, GmMYB84, and GmDHN15 in GmPYR1-OE plants and in the ABFs transgenic soybean hairy roots lines. The results indicated that the expression levels of the GmRD22, GmNAC4, GmMYB84, and GmDHN15 stress-responsive genes were all higher in GmPYR1-OE plants and ABF-overexpressing soybean hairy roots than that in WT under drought and salt conditions (Figure S9c–d), suggesting that these four stress-responsive genes could be downstream regulatory targets of GmPYR1 and ABFs.

To further assess the interactions among GmRD22, GmNAC4, GmMYB84, and GmDHN15 and the four ABFs, we cloned the promoters of these target genes into the pGreenII0800 vector containing a LUC reporter. Analysis of LUC activity showed that all four target promoters drove expression of a high LUC signal in the presence of ABF proteins (Figure S9f–g). Further analysis of these promoter sequences revealed the presence of ACCTG cis-acting elements, which are known to bind to ABF proteins (Figure S10a). To determine whether this motif was relevant to ABF recognition of these targets, we next induced the expression of ABF proteins
and purified them in vitro for EMSAs with a biotin-labelled probe based on the respective promoter sequences of GmRD22, GmNAC4, GmMYB84, and GmDHN15. We observed that each of the ABF proteins could directly bind to the ACGTG cis-acting elements of these four genes promoters (Figure 7a). These results together demonstrated that the expression of GmRD22,
GmNAC4, GmMYB84, and GmDHN15 was regulated by ABF transcription factors.

**Soybean PYR1-mediated regulation of the ABA signal transduction pathway**

ABA signal transduction is mediated by the PYR receptor protein, which regulates the phosphorylation activity of SnRK2s and affects the regulation ability of ABF transcription factors (Zhu, 2012). To investigate GmPYR1-mediated regulation of ABA signalling, we first detected changes in the phosphorylation of ABFs in the absence or presence of GmPYR1 protein. Firstly, we transiently expressed ABF-GFP fusion proteins in tobacco leaves (Figure 7b). Phos-tag assays confirmed that GFP-tag was not phosphorylated in tobacco leaves (Figure 7c), whereas the ABF-GFP fusion proteins were phosphorylated following 2 h of ABA treatment (Figure 7d). After 4 h of ABA treatment, no significant

**Figure 6** Drought tolerance of GmPYR1-overexpressing soybean plants. (a) Phenotypic analysis of GmPYR1-OE adult soybean plants under drought stress, (b–f) Number of pods per plant (b); plant height (c); stem base circumference (d); grain number per plant (e); and grain weight per plant (f) of GmPYR1-OE and WT soybean plants under drought stress conditions. (g) Phenotypic analysis of GmPYR1-OE seeds under conditions of drought stress at the adult stage. (h) Relative content of different seed types of the GmPYR1-OE and WT soybean plants under drought conditions at the adult stage.

**Figure 7** Interaction between the soybean PYR1 protein and ABF transcription factors. (a) EMSA identified the interaction between the ABF transcription factor and four stress-responsive genes. (b) ABF proteins expressed in tobacco leaves. (c) Empty vector GFP-tag proteins were expressed in tobacco leaves and lacked phosphorylation activity as evidenced in the Phos-tag assay. (d–f) Phos-tag assay of ABF proteins after 2 h (d, e) and 4 h (f) of ABA treatment. (g, h) Expression of ABF proteins in tobacco leaves after 2 and 4 h of ABA treatment. (i, j) Phosphorylation activity of ABF proteins after 2 h and 4 h of ABA treatment when soybean PYR1 was transformed into tobacco leaves. (k, l) Expression of ABF proteins after co-transformation in tobacco leaves with soybean PYR1 protein. (m–p) Relative LUC activity of the interactions between soybean PYR1 and ABFs under ABA treatment. PP: λ protein phosphatase....
NF-Y transcription factor confers drought and salt tolerance in soybean

(a, b, c, d, e, f, g, h, i, j, k, l, m, n, o, p)
Differences were observed in the phosphorylation of ABF-GFP proteins compared with that observed at 2 h (Figures 7e-f). When GmPYR1-Flag and ABF-GFP were co-expressed in tobacco leaves, the phosphorylation of ABF proteins increased significantly and stabilized at high levels throughout 4 h of ABA treatment in the presence of GmPYR1 (Figures 7i,j). These results thus indicated that GmPYR1 mediated the phosphorylation of ABF transcription factors.

To evaluate the interaction between GmPYR1 and ABF, the promoters of stress-responsive genes (proGmRD22, proGmDHN15, and proGmNAC4) that we found could interact with ABF proteins were selected as markers. In leaves of Nicotiana benthamiana, injection with GmPYR1 resulted in increased ABA-mediated up-regulation of LUC signal, driven by stress-responsive gene promoters (proGmRD22, proGmDHN15, and proGmNAC4) (Figure S10b,c). Furthermore, the LUC signal that driven by the stress-responsive promoters (proGmRD22, proGmDHN15, and proGmNAC4) was further increased when the GmPYR1 protein was co-injected with each of these ABA transcription factors under ABA treatment (Figures 7m–p, Figure S10d,e). These results supported the hypothesis that GmPYR1 could regulate the ABA signal transduction pathway.

Discussion

The B and C subunits of the NF-Y transcription factor complexes are known to be essential for DNA binding and bending; in particular, NF-YC harbours an important secondary structure element, an α helix, which participates in NF-YA binding and affects growth factor signalling (Romier et al., 2003). The NF-YC subunit can form a heterotrimeric complex with other (i.e. non-NF-Y) proteins to regulate different signalling pathways. For example, NF-YC forms a heterotrimer with CONSTANS and NF-YB which controls plant flowering (Gnesutta et al., 2017). Moreover, NF-YC interacts with at least two different zinc finger transcription factors to form respective nuclear complexes that regulate ABA signalling or the expression of genes associated with endoplasmic reticulum-related stress (Liu and Howell, 2010; Yamamoto et al., 2009). Thus, NF-YC-mediated regulation is essential for plant growth and development. Here, we identified members of NF-YC transcription factor family by analysing de novo transcriptome data obtained under plant exposure to drought conditions (Figure S1,S2). In particular, the GmNF-YC14 gene was screened by analysing the expression patterns of soybean NF-YC family members (Figure S3). Furthermore, we assessed the correlation between soybean NF-YC gene expression and abiotic stress responses.

Drought stress induces synthesis of the plant hormone ABA, and increased levels of ABA induce stomatal closure to reduce water loss (Zhu, 2016), thereby enhancing plant adaptation to drought conditions. Our present study showed that under stress-inducing conditions, overexpression of GmNF-YC14 in Arabidopsis and soybean enhances the tolerance of transgenic plants to drought and salt stresses, as well as sensitivity to ABA (Figure 5A, Figures 1 and 2). These findings indicate presence of a relationship between the NF-YC transcription factor complex, ABA, and plant stress tolerance. The ABA signalling pathway is composed of two parts, that is, the ABA biosynthetic pathway and the ABA signal transduction pathway. De novo transcriptome analysis revealed that transcript levels of the ABA receptor PYR/PYL (GmPYR1) – a component of the ABA signal transduction pathway – were enhanced in soybean plants overexpressing the GmNF-YC14 gene (Figure 4), suggesting that the GmNF-YC14 gene can regulate PYR1-mediated ABA signal transduction. However, to regulate the expression of downstream effector genes, NF-YC proteins usually interact with other transcription factors or form heterotrimer with NF-YA and NF-YB (Ceribelli et al., 2008; Frontini et al., 2004; Kahle et al., 2005; Peng, 2003; Yiwen and Nadia, 2002). Indeed, we identified two other NF-Y transcription factor subunits (GmNF-YA16 and GmNF-YB2) by screening a soybean cDNA library and subsequently observed interactions between these three subunits. Therefore, our study identified a novel NF-YC/A-PYR/PYL module through which NF-Y transcription factors enhance ABA signal transduction in soybean via transcriptional regulation of PYR1 (Figure 8).

The ABA receptor PYR/PYL binds ABA molecules to form an initiation complex (Yue et al., 2009; Zhu, 2016). This complex interacts with protein phosphatase 2C (PP2C) which is associated with SnRK2s kinases, thus ensuring these kinases remain inactive (Soon et al., 2012). The combination of PYR/PYL-ABA complex and PP2C promotes the release of SnRK2 from the PP2C-SnRK2 complex, and the released SnRK2 then phosphorylates downstream ABF transcription factors (Fujii and Zhu, 2009). Recent research has shown that overexpression of PYR/PYL in wheat confers drought tolerance, indicating a relationship between PYR/PYL and drought tolerance (Mega et al., 2017). Our research reveals that an increase in soybean PYR1 protein levels results in enhanced phosphorylation and regulatory activities of ABF transcription factors (Figure 7). Our results also show that PYR/PYL overexpression in soybean confers higher tolerance to drought and salt stress.
drought and salt stresses and promotes the expression of stress-responsive gene. This up-regulation of stress-responsive genes improves plant stress tolerance by regulating the activity of ABF transcription factor.

ABF transcription factors play important roles in mediating abiotic stress responses and are important regulatory factors in the ABA signal pathway. For example, overexpression of AtABF3 or AtABF4 in Arabidopsis enhances stress tolerance (Kang, 2002). Notably, both AtABF3 and AtABF4 promote stomatal closure and improve seedling survival rates under water-deficit conditions (Chung et al., 2009; S. Kim et al., 2010). Overexpression of PtA 8 BF in Poncirus trifoliata results in enhanced dehydration tolerance, reduced stomatal density in leaves, and maintenance of reactive oxygen species (ROS) homeostasis (Zhang et al., 2015). These findings suggest that ABF transcription factors can regulate stomatal closure and density under water-deficit conditions. Indeed, we found that overexpression of GmNF-YC14 enhances stomatal closure under ABA treatment. Moreover, we found that GmNF-YC14 overexpression results in enhanced expression of ABF transcription factors (ABF1-ABF4). However, further analysis revealed that CCAAT cis-acting elements recognized by NF-Y transcription factor are present in the promoters of ABF2, ABF3, and ABF4, but not in the promoter of ABF1. Therefore, we postulated that overexpression of GmNF-YC14 in soybean indirectly regulates ABF1 expression under stress conditions, and moreover, GmNF-YC14 regulates stomatal closure by mediating ABF transcription factor activity, either directly or indirectly.

We also found that overexpression of GmNF-YC14 enhances the expression of the stress-responsive genes GmDHN15, GmMYB84, GmNAC4, and GmRD22 (Figure 4). Among these genes, GmNAC4 promotes auxin signalling, and its overexpression in Arabidopsis counteracts the ABA-induced inhibition of root system development to enhance the root system growth under water-deficit conditions (Quach et al., 2014). GmRD22 regulates the activity of cell wall peroxidases and thus strengthens cell wall integrity under stress conditions, a phenomenon that can enhance plant resistance to drought and salinity stresses (Wang et al., 2012). GmDHN15, a dehydrin family gene, is a key contributor to stress tolerance, and the studies have reported that dehydrin family members can respond to abiotic stresses in plants. For example, two soybean Ks-type dehydrin genes, SLT166 and SLT1629, were found to confer tolerance against osmotic and metal stresses in E. coli and Arabidopsis (Chung et al., 2009). The expression of GmMYB84—a MYB transcription factor with diverse reported roles in plant development and stress responses—is up-regulated in response to drought and exposure to exogenous ABA, and has been reported to confer drought tolerance in transgenic soybean plants (Wang et al., 2017). ROS have been reported to regulate the growth of plant cells (Foreman et al., 2003), and the study found that interactions between GmMYB84, ROS, and antioxidant enzymes can promote root growth under drought conditions (Wang et al., 2017). Moreover, the expressions of these four genes are regulated by the ABA pathway. Further analysis of GmPYR1 and ABF function in the current study revealed that the NF-Y complex regulates the transcription of stress-responsive genes by directly or indirectly enhancing ABA signalling (Figure 8).

Overall, the findings of our study indicated that the novel NF-YC/BA1-IPYR/PYL regulatory module—an NF-Y transcription factor complex—can improve plant tolerance of abiotic stress by regulating the ABA pathway. Moreover, we show that the GmNF-YC14 and GmPYR1 gene can improve the agronomic traits of transgenic soybean plants under drought conditions, highlighting their potential value in the creation of new drought tolerance varieties, which can provide social benefits through increased food security.

Methods

Plant materials and qPCR analysis

The soybean cultivar ‘Williams 82’ was used to isolate NF-YCs and evaluate their expression patterns under stress conditions at different time intervals (0, 0.5, 1, 2, 5, 12, and 24 h). Soybean seedlings (four-leaf stage) were exposed to salt, dehydration, cold, and ABA. To induce salt stress, seedling roots were dipped in 200 mM NaCl; to stimulate cold stress conditions, seedlings were placed in a 4 °C growth chamber; for dehydration, the root systems of whole plants were washed gently with water to remove soil and then the plants were placed on filter paper to induce drought; to induce ABA stimulation, soybean seedlings were sprayed with 200 μM ABA. After each treatment, soybean seedlings were frozen in liquid nitrogen and stored at −80 °C for subsequent analyses. Real-time PCR was performed using SYBR green qPCR Master Mix (Tiangen, Beijing, China) on a 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA). Relative expression was determined using the 2^{-ΔΔCT} method based on CT values (Livak and Schmittgen, 2001). All primers used in the study are listed in Table S4.

Isolation of GmNF-YC14 gene and yeast two-hybrid screening assays

Total RNA was extracted with the RNeasy Prep Pure Plant Kit (Tiangen) and reverse transcribed to cDNA using PrimeScript™ Reverse Transcriptase (Takara Bio, Shiga, Japan). GmNF-YC14 was cloned from cDNA using the specific primers listed in Table S5. The resulting PCR product was purified and cloned into the pEASY-blunt Vector (TransGen Biotech, Beijing, China) for sequencing. Multiple sequences were aligned using DNAMAN software.

The CDS of GmNF-YC14 was cloned into pGBK7 vector at the Ncol site using specific primers. Transformation was performed using PEG/ LiAc yeast as described in the Yeast Protocols Handbook PT3024-1 (Takara Bio). Competent cells of yeast strain AH1109 transformed with pGBK7-GmNF-YC14 and pGADT7 were used as the negative control. The resulting diploid yeast cells were selected on SD media, either without leucine and tryptophan or without histidine and adenine, and incubated at 30 °C for 3–5 days. Interaction between the bait protein and candidate protein was determined on synthetic complete medium lacking Trp/Leu/His/Ade.

LCI assay

The firefly LCI transient expression assay was performed as previously described (Chen et al., 2008; Fujikawa and Kato, 2007). The open reading frames (ORFs) of GmNF-YC14, GmNF-YA16, and GmNF-YB2 were cloned into pCAMBIA1300-nLUC or pCAMBIA1300-cLUC vectors. Agrobacterium GV3101 carrying the nLUC and cLUC derivative binary plasmids (GmNF-YC14-nLUC and GmNF-YB2-cLUC; GmNF-YC14-nLUC and GmNF-YA16-cLUC; GmNF-YB2-nLUC and GmNF-YA16-cLUC) was separately co-injected into leaves of 4-week-old Nicotiana benthamiana. Plant leaves with corresponding empty vectors pCAMBIA1300-nLUC and pCAMBIA1300-cLUC were used as negative controls. Luciferase activity was measured 48 h after
generation with a low-light cooled CCD imaging apparatus (Night SHADE LB 985; Berthold Technologies, Bad Wildbad, Germany).

**BIFC assay**

The ORFs of GmNF-YC14, GmNF-YA16, and GmNF-YB2 were cloned into the pUC-SPYNE and pUC-SPYCE vectors. The vectors (pSPYNE-GmNF-YC14 and pSPYCE-GmNF-YA16; pSPYNE-GmNF-YB2 and pSPYCE-GmNF-YA16) were co-transfected into Arabidopsis protoplasts by PEG-mediated transfection and the protoplasts were then incubated in the dark at 22 °C, using a protocol proposed by Yoo et al. (2007). YFP fluorescence of the protoplasts was observed with a confocal laser scanning microscope (LSM 700; Zeiss, Oberkochen, Germany).

**In vitro GST pull-down assays**

To evaluate the interaction between GmNF-C14 and GmNF-YA16 in vitro, the coding region of GmNF-YA16 was cloned into the pCold™ TF DNA vector (TaKaRa Bio) at the BamHI site to generate a His-pCold-GmNF-YA16 protein expression construct. In the pGEX-4T-1 vector (GE Healthcare, Chicago, IL), a GST tag was fused with GmNF-C14 at the BamHI site, generating the GST-GEX-GmNF-YC14 fusion protein expression construct. Two constructs (His-pCold-GmNF-YA16 and GST-GEX-GmNF-YC14) were introduced into E. coli strain transetta (TransGen Biotech), and expression of the corresponding proteins was induced overnight using 0.5 mM isopropyl β-D-thiogalactoside (IPTG) (Nalco SPA, San Luis Obispo, CA) at 16 °C. His-pCold-protein and GST-GEX-GmNF-YC14 were purified using Ni-NTA agarose beads and glutathione agarose beads, respectively (TransGen Biotech); then, the His-pCold-protein was eluted with His elution buffer (500 mM imidazole). Finally, the eluted His-pCold-protein was added to glutathione agarose beads that adsorbed GST-GEX-GmNF-YC14. The pull-down reaction was rotated overnight at 4 °C and washed five times with washing buffer. Anti-His antibody (TransGen Biotech) was used for the immunoblot.

**Generation of transgenic Arabidopsis plants and identification of stress tolerance**

To construct an expression vector for Arabidopsis, the ORF of GmNF-YC14 was cloned into the pEASY-Blunt vector, and then subcloned into pBl121 (under control of the CaMV 35S promoter). Arabidopsis plants were transformed using the floral dip method. Transformants were selected on MS medium containing 50 mg/mL kanamycin, and T3 generation plants were used for further analyses. Arabidopsis plant transformation was performed per the protocol described by Yu et al. (2017). Phenotype was determined using 2-week-old Arabidopsis plants grown under 16-h illumination with an average daytime temperature of 23 °C. To induce drought conditions, water was withheld for 2 weeks, followed by full re-watering and recovery. To induce salt stress, plants were irrigated with 250 mM NaCl and then transferred to pots under normal growth conditions. The survival rate was calculated 2 weeks later.

**Stomatal aperture and measurement of water loss rate**

Rosette leaves of 4-week-old WT plants and GmNF-YC14 transgenic lines were detached, incubated for 4 h in stomata-opening solution (10 mM MES [pH 6.1], 100 mM CaCl₂, and 10 mM KCl), and then transferred into ABA-containing solution for 2 h for stomatal aperture analysis, as described by Kim and Kim (2013). To determine the rate of water loss, leaves excised from 4-week-old WT plants and GmNF-YC14 transgenic lines were placed on a bench at 30% relative humidity, and the fresh weight of leaves was measured at specific timepoints, as described by Yu et al. (2017).

**Generation of different types of transgenic soybean plants**

The CDS of GmNF-YC14 was amplified and ligated into the pTF101 vector to generate the recombinant vector GmNF-YC14-pTF101. GmNF-YC14-pTF101 and the expression vector CRISPR/Cas9-GmNF-YC14 were then transformed into Agrobacterium strain EHA105 (Zoman Biotechnology, Beijing, China). The soybean variety ‘Williams 82’ was used as the recipient material for transformation, and stable transformation was performed based on the protocol described by Chen et al. (2018). Mutant plants generated by CRISPR/Cas9 were screened by DNA sequencing (Figure S5a), and the GmNF-YC14 transgenic soybean plants were confirmed by qPCR (Figure S5b,c). Briefly, DNA was extracted from leaves of the T0 generation of each individual plant, and genomic regions spanning the target sites were amplified by PCR using rTaqMix (TaKaRa Bio). PCR products were sequenced to identify gene editing events. Base insertions or deletions induced by CRISPR/Cas9 could lead to translational frame shift mutations.

**Phenotype identification of different types of soybean plants**

T3 homozygous mutant, WT, and overexpressing soybean plants were used to evaluate stress tolerance. Soybean seeds were sown in flowerpots of the same weight and grown under normal conditions (16 light/8 dark, 25 °C, 50% humidity). Three-week-old seedlings were then treated with 300 mM NaCl or subjected to drought conditions. After 3 and 5 days of salt and drought stresses, respectively, the leaves were collected for qPCR, and physiological and biochemical index analyses. Plants were also analysed at the flowering stage; for inducing salt stress, the plants were exposed to 500 mM NaCl, and for inducing drought stress, watering was controlled to maintain soil water content within 9.5%–9.8% at the bottom of the pot (Table S6,7). After 3 and 5 days of salt and drought stresses, respectively, the leaves were collected for qPCR, and physiological and biochemical index analyses. For adult plants, seeds were sown into field soil and grown under normal condition. We controlled plant watering to maintain soil water content at different soil depths (0-20 cm and 20-40 cm) within 8.7%–9.5% to recapitulate drought conditions (Table S8). After harvest, the agronomic traits of different soybean plant types were determined and subjected to statistical analysis. All experiments were designed with three independent replicates.

**Transformation and detection of soybean hairy roots**

Functional genes (GmNF-YA16, GmNF-YB2, and ABFs) were amplified by PCR and cloned into pCambia3301 vector. The recombinant vectors were transferred into Agrobacterium rhizogenes strain K599 and transformed into soybean cotyledons hypotolsts by A. rhizogenes to form hairy roots. The true roots of soybean plants were excised, and then soybean plants were grown with hairy roots to form transgenic soybean hairy root composite plants, as described by Kereszt et al. (2007) and Sun et al. (2015). Soybean cotyledon hypocotyls can usually generate three to five soybean hairy roots overexpressing the functional genes. qPCR using gene-specific primers (Table S9) confirmed
that the functional genes were overexpressed in the hairy roots (Figure S9).

To evaluate drought and salt tolerance of composite transgenic soybean plants, the plants were treated with 250 mM NaCl or subjected to drought. After 5 days of stress, the phenotype was recorded, and the plants were removed from the soil. The hairy roots were quickly removed and stored in liquid nitrogen. Finally, the transgenic soybean hairy roots were analysed with qPCR, and positive soybean hairy roots were used for physiological and biochemical index analysis.

**LUC activity analysis**

To analyse protein–protein interactions, the candidate protein (GmNF-YA1A and GmNF-YB2) and bait protein (GmNF-Y C14) were cloned into vectors containing cLUC and nLUC tags, respectively. The recombinant vectors were then transformed into competent cells of the A. rhizogenes GV3101 strain (Zoman Biotechnology). The recombinant and control blank vectors were co-expressed in N. benthamiana leaves for 3 days via A. rhizogenes-mediated transformation. After 3 days of growth, the N. benthamiana leaves were removed, and their surfaces were treated with D-luciferin (Biovision, Milpitas, CA). After 5 min in the dark, LUC signal was observed using a low-light cooled CCD imaging apparatus (Night SHADE LB 985).

To determine transcriptional activity, genes encoding transcription factors (TFs) were cloned into pCambia1305-GFP and target gene promoters were cloned into pGreen0800II. Then, the recombinant and control blank vectors were co-expressed in N. benthamiana leaves and grown for 3 days, after which the leaves were removed, and their surfaces were treated with D-luciferin. After 5 min in the dark, LUC signal was observed using a plant living imaging system. Total protein in the leaves was extracted using the Plant Total Protein Extraction Kit (CoWin Biosciences, Boston, MA), and the proteins were detected using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI). LUC data were collected using an automatic microplate reader.

**In vitro EMSA binding assay**

An EMSA was performed to verify binding between TFs and cis-acting elements from the target gene promoters. The TF genes were cloned into expression vectors containing the tag proteins (His, GST, and MBP) and transferred into competent cells of the E. coli strain Rosetta. Expression of TFs was induced with IPTG, and eluted proteins were purified using protein purification resins and eluted with tag protein elution buffer. The eluted proteins and biotin end-labelled duplex DNA probe were mixed with EMSA binding buffer, incubated at 25 °C for 30 min to allow the TF proteins to interact with the probe, and then resolved by native gel electrophoresis using 5x-TBE. Then, the protein–DNA complexes were transferred rapidly from native gel to a positive soybean KS-type dehydrin, SLTI66 and SLTI629 conferred tolerance against osmotic and metal stresses of Escherichia coli and Arabidopsis. Chen, J., Ying, P., Miao, Y., Xing, D., Wang, Y., and Hou, W. (2018) Improvement of Soybean Agrobacterium-Mediated Transformation Efficiency by Adding Glutamine and Asparagine into the Culture Media. International Journal of Molecular Sciences, 19(10), 3039. Cheng, M.C., Liao, P.M., Kuo, W.W. and Lin, T.P. (2013) The arabidopsis ethylene response factor1 regulates abiotic stress-responsive gene expression by binding to different cis-acting elements in response to different stress signals. Plant Physiol, 162, 1566–1582.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1 Transcripctome sequencing analysis of drought induced soybean plants.
Figure S2 Phylogenetic relationships of NF-YCs with soybean, Arabidopsis and rice.
Figure S3 Expression pattern analysis of soybean NF-YC family genes under different stress conditions.
Figure S4 Expression patterns of GmNF-YC14 and identification of stress tolerance.
Figure S5 The identification of GmNF-YC14 transgenic soybean plants.
**Figure S6** GmNF-YC14-overexpressing plants have a stronger root system than WT and mutant plants under stress conditions.

**Figure S7** Identifying interaction and subcellular localization analysis among GmNF-YA16, GmNF-YB2, and GmNF-YC14 proteins.

**Figure S8** Detection of positive transgenic soybean hairy roots and transgenic soybean plants.

**Figure S9** Analysis of the interaction between ABF transcription factors and four stress-responsive genes.

**Figure S10** Relationships between soybean PYR1 protein and ABF transcription factor activity.

**Figure S11** Binding analysis of tag protein to cis-acting elements from the promoters of stress-responsive genes.

**Table S1** Different types of seeds divided into four groups (field).

**Table S2** Differential expression data of functional genes in the transgenic soybean.

**Table S3** Gene identification of the functional genes.

**Table S4** qPCR primers for soybean NF-YC family genes.

**Table S5** GmNF-YC14 gene-specific primers used in this article.

**Table S6 and S7** Soil water content during the seedling stage (flowerpot).

**Table S8** Soil water content during the seedling stage (field).

**Table S9** qPCR primers for stress-related genes.

**Table S10** The specific primers of transgenic soybean detection.

**Table S11** The related primers of ABF genes.

**Table S12** Primers of stress-related gene promoters.