Overexpression of GmNFYA5 confers drought tolerance to transgenic Arabidopsis and soybean plants

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Overexpression of *GmNFYA5* confers drought tolerance to transgenic *Arabidopsis* and soybean plants

Running title: Drought tolerance of transgenic plants is up-regulated by *GmNFYA5*

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Abstract

Background: The crop productivity is challenged by abiotic stresses, among which drought stress is the most widespread. The NF-Y genes, especially NF-YA genes function in regulating drought tolerance of plants.

Results: In this study, a soybean NF-Y gene, *GmNFYA5*, was identified with the highest transcript level among all of 21 *NF-YA* genes in soybean (*Glycine max* L.) under drought stress. Transcript of *GmNFYA5* induced by drought was suppressed by ABA synthesis inhibitor naproxen (NAP). *GmNFYA5* transcript was detected in various tissues at vegetative and reproductive growth stage with higher levels in roots and leaves, which was consist with the *GmNFYA5* promoter:GUS fusion assay. Overexpression of *GmNFYA5* resulted in increased drought tolerance and ABA sensitivity in transgenic *Arabidopsis* plants. Additionally, overexpression and suppression of *GmNFYA5* in soybean resulted in increased and decreased drought tolerance compared with empty vector (EV) plants respectively. Transcript levels of ABA-dependent and ABA-independent genes in transgenic *Arabidopsis* and soybean plants overexpressing *GmNFYA5* were higher than those of WT and EV plants under drought stress respectively, but the opposite results were detected in soybean plants suppressing *GmNFYA5*. Furthermore, GmNFYA5 might regulate the expression abundance of *GmDREB2* and *GmbZIP1* by binding the promoter in vivo.

Conclusions: These results suggest that overexpression of *GmNFYA5* can improve drought tolerance via ABA-dependent and ABA-independent pathways in soybean.

Keywords: Nuclear Factor YA, drought tolerance, ABA sensitivity, resistant mechanism, soybean

Background

Plant agriculture is afflicted by abiotic stresses, which lead to soil destruction and crop loss worldwide [1, 2]. Water stress is a major challenge, containing both drought and salt stresses; compared to salt stress, drought is more widespread and damaging [3]. The adaptation to drought involves complex regulatory networks containing

control of water flux and cellular osmotic adjustment in plants [4-6]. Some transcription factor genes were known to play an important role under stress, such as AP2/ERF and MYB family [7-9].

The CCAAT box binding factor (CBF), also known as Nuclear Factor Y (NF-Y) transcription factor or Heme Activator Protein (HAP), which is composed of three subunits: NF-YA, NF-YB and NF-YC [10-12]. In monocotyledonous and dicotyledonous plants, one subunit is encoded by tens of genes, but by only one or two genes in animals [11]. The members of different NF-Y families have diverse functions in regulating plant development and growth, such as flowering time, plant height, root elongation, embryogenesis, seed germination [13-24].

The NF-Y genes are excellent regulators of abiotic stress-induced responses. Overexpression of *AtNFYA5* in *Arabidopsis* resulted in enhanced resistance to drought stress by affecting the expression of many drought stress related genes [25]. Transgenic rice lines of *OsNFYA7* could improve the level of drought tolerance via ABA-independent pathway [26]. Transgenic rice overexpressing *OsHAP2E* [27] and bermudagrass *Cdt-NFYC1* [28] displayed improved resistant to drought and salt stresses. A lot of stress-related parameters showed that overexpression of *ZmNFYB2* resulted in drought resistance to transgenic maize plants [29]. Most reports showing that NF-Ys play an important role in water stress mainly focused on rice and *Arabidopsis*. The function of a very few NF-Y members was confirmed in soybean, *GmNFYA3* was found to be induced by drought and the transgenic *Arabidopsis* lines showed increased tolerance to drought stress [30]. However, the biological function of many other NF-Y members remains to be verified in soybean.

In this study, *GmNFYA5*, a member of the NF-YA family in soybean, was induced by drought and ABA. The transgenic *Arabidopsis* and soybean lines with positive hairy roots overexpressing *GmNFYA5* showed enhanced tolerant to drought stress in ABA-dependent and ABA-independent pathways.

Results

Isolation and characterization of *GmNFYA5*

It was reported that the NF-YA genes mediated in drought stress tolerance [25, 26, 30]. 21 NF-YA genes were investigated in seedling leaves using qRT-PCR, most of which were induced by drought stress. Transcript of *GmNFYA5* was highest among all of the 21 genes at 2 h after drought stress (Figure 1A). Also, transcript of *GmNFYA5* was induced by ABA significantly (Figure 1B). Detached leaves were used to understand whether ABA could affect the drought-induced transcript of *GmNFYA5* by using NAP, which is an inhibitor of ABA biosynthesis. The *GmNFYA5* transcript under drought treatment for 2 h was similar to the above pattern compared with the control (Figure 1A and Figure 1C). However, pretreatment with NAP suppressed the expression level of *GmNFYA5* (Figure 1C).

GmNFYA5 encodes a peptide of 303 amino acid residues with a polypeptide of 37.68 KD and an isoelectric point (p*I*) of 8.86. GmNFYA5 contains highly core regions common to *Arabidopsis* NF-YA proteins which are consists of two subdomains: a NF-YB/C binding subdomain and a DNA binding subdomain, connected by a linker and required for association with the NF-YB/C heterodimer and binding CCAAT sequences respectively. The amino acids indicated by asterisks are critical residues and conserved. Alignments of GmNFYA5 and *Arabidopsis* NF-YA proteins in the conserved domain showed that it had the highest identity with AtNFYA5 (Figure S1A). Phylogenetic analysis based on the amino acid sequences in conserved domain showed that GmNFYA5 clustered with AtNFYA6 and AtNFYA5 in *Arabidopsis* (Figure S1B).

Tissue specific expression analysis and subcellular localization

Under normal conditions, the expression of *GmNFYA5* in different tissues, including roots, stems, cotyledon, leaves, apical buds, flower buds and flower were evaluated by qRT-PCR at vegetative and reproductive growth stage. The transcript of *GmNFYA5* was detected in every tissue at two developmental stages, with a significantly increase in leaves at flowering stage compared with seedling stage, and the transcript abundance in roots was very high compared with that in other tissues. To analysis the expression patterns in greater detail, transgenic *Arabidopsis* T3 lines overexpressing

GmNFYA5 promoter:GUS were researched. The results showed that the expression of GUS was detected in various tissues (Figure 2B). The level of *GmNFYA5* expression was high in floral tissues, with prominent expression in the leaf vascular system and roots. At seedling stage, a low level of GUS staining was observed in leaves, with a higher level of staining at flowering stage (Figure 2B), which were consistent with the results of the qRT-PCR.

To investigate the localization of GmNFYA5 in cells, recombinant vectors p16318GFP:*GmNFYA5* and mCherry:*GmNFYA3* were co-transformed into *Arabidopsis* protoplasts. GFP and mCherry fluorescence of two fusion protein in transformed cells were localized exclusively to the nucleus collectively (Figure 2D). Control GFP was uniformly distributed all over the protoplasts cell (Figure 2C), which showed that GmNFYA5 was a nuclear-localized protein.

Sensitivity of Transgenic *Arabidopsis* Plants to Exogenous ABA and PEG

To measure ABA sensitivity as affected by *GmNFYA5*, seeds from transgenic and WT plants were germinated on 1/2 MS medium with different concentrations of ABA. The germination rate of seeds was similar in all plants under control conditions (Figure 3A). In the presence of 0.5 and 0.8 μM ABA, seed germination was suppressed significantly in transgenic *Arabidopsis* and WT lines, the inhibition of WT seeds was much smaller than transgenic seeds. Germination rate of WT seeds reached up to 73.15% at 36 h compared with only 12.04 to 18.52% germination rate in transgenic lines at 0.5 μM ABA (Figure 3A). Only 7.41 to 12.96% germination rates were detected in transgenic lines compared to 62.04% germination rate of the WT seeds by 36 h at 0.8 μM ABA (Figure 3A).

Inhibition of root growth between WT and transgenic seedlings was also different by ABA. Three-day-old seedlings under normal conditions were transferred to1/2 MS medium with 0.5-1 μM ABA. A week later the length of roots was measured, all seedlings had similar root length under normal conditions, while the length of roots in transgenic plants was shorter than WT plants when treated with ABA (Figure 3B and Figure 3C).

In addition, PEG sensitivity of seeds in WT and transgenic lines was measured by

being germinated on 1/2 MS medium with different concentrations of PEG. The germination of WT seeds was more sensitive to PEG than transgenic lines. Compared to 53.06-58.5% germination rates of the transgenic seeds by 48 h, only 25.17% germination rates were observed in WT seeds when treated with 8% PEG (Figure 4A). Besides, WT seeds germinated much later than those of transgenic seeds under the treatment of 10% PEG (Figure 4A).

Growth of seedlings was also inhibited differentially by PEG in WT and transgenic lines. Three-day-old seedlings were transferred to 1/2 MS medium with 10-12% PEG and measured in root length a week later. Compared to almost equal root length in all plants under control conditions, the root of transgenic plants was longer than those of the WT in presence of PEG (Figure 4B and Figure 4C).

Tolerance analysis of transgenic *Arabidopsis* plants to drought stress

Transgenic *Arabidopsis* plants overexpressing *GmNFYA5* were generated to elucidate the role of the gene. Three homozygous transgenic lines (OE-1, OE-2 and OE-5) detected using qRT-PCR were selected for functional analysis (Figure 5B). WT plants appeared to be extreme wilting by withholding water for 14 days, while the transgenic lines still displayed turgid (Figure 5A). After re-watering for 7 days, a majority of WT plants were unable to recover (19.44% survival). By contrast, most transgenic lines still displayed turgid (55.56-86.11% survival) as showed in Figure 5C. Decreased RWC and increased ion leakage were caused by drought stress in all plants. Transgenic lines maintained 64.98-69.37% RWC and 54.31-62.42% ion leakage compared to 24.34% RWC and 79.29% ion leakage in leaves of WT plants (Figure 5E and Figure5F). Moreover, detached leaves of transgenic plants lost water more slowly than those of WT (Figure 5D).

The concentrations of ABA in transgenic lines were higher than those of WT plants under drought condition with similar concentrations of ABA between WT and transgenic lines under normal condition (Figure 5G). Under the treatment of 10 μM ABA, the stomatal aperture of transgenic lines was smaller significantly than that of WT and no significant difference was observed between WT and transgenic lines under normal condition (Figure 5H and Figure 5I).

Transcription profiles of stress-related genes in transgenic *Arabidopsis*

To research the role of *GmNFYA5* in regulation of drought stress tolerance, 9 stress-related genes including 6 ABA-dependent genes (*ABI2*, *ABI3*, *NCED3*, *LEA3*, *RD29A* and *P5CS1*) and 3 ABA-independent genes (*DREB1A*, *DREB2A* and *DREB2B*) were analyzed via using three transgenic lines in comparison with WT plant under treatments of control, drought and drought pretreated with NAP. Under control condition, the transcripts of *DREB1A*, *DREB2A*, *DREB2B*, *ABI2*, *ABI3*, *LEA3*, *RD29A* and *P5CS1* in transgenic plants were significantly higher than those in WT plants, while *NCED3* transcript exhibited no significant difference (Figure 6). Moreover, all genes maintained higher transcript levels in transgenic *Arabidopsis* plants than those in the WT plants under drought stress (Figure 6). However, NAP suppressed the drought-induced transcripts of all genes, especially *ABI2*, *ABI3*, *LEA3*, *RD29A* and *P5CS1* (Figure 6E-I).

Tolerance analysis of transgenic soybean plants to drought stress

Three transgenic soybean lines OE, EV and RNAi were generated to research the functions of *GmNFYA5* by *A. rhizogenes*-mediated transformation of soybean hairy roots. Results of qRT-PCR showed that the relative mRNA level of *GmNFYA5* in OE plants is higher significantly than that of EV. Significantly higher level of *GmNFYA5* transcript was observed in EV plants than that in RNAi plants.

The RNAi plants displayed severe wilting by withholding water for 16 days, while a small part of EV plants and a majority of OE plants appeared to be healthy (Figure 7b). After re-watering for 3 days, the ratio of OE plants survived was larger significantly than that of EV plants, nearly all of the RNAi plants were unable to recover eventually (Figure 7C and Figure 7H). Increased ion leakage, MDA and decreased RWC were observed with the treatment of drought in all plants. Higher RWC and lower ion leakage and MDA were detected in OE plants compared with those of EV plants, and the opposite results were observed in RNAi plants (Figure 7E-G). Water loss of the leaves was unable to be observed because the three transgenic soybean lines only had positive hairy roots.

Transcription profiles of stress-related genes in transgenic soybean lines

The transcript levels of 6 stress-responsive genes including 3 ABA-dependent genes (*GmDREB1*, *GmDREB2* and *GmDREB3*) and 3 ABA-independent genes (*GmWRKY46*, *GmNCED2* and *GmbZIP1*) were analyzed using qRT-PCR with treatments of control, drought and drought pretreated with NAP. Under control condition, significantly higher expression levels of *GmDREB1*, *GmDREB2*, *GmDREB3*, *GmWRKY46* and *GmbZIP1* were detected in OE soybean hairy roots, the opposite results were observed in RNAi lines compared with those of EV lines, while *GmNCED2* transcript showed no significant difference (Figure 8A-F). Drought induced expression levels of the 6 genes which showed significantly higher in OE soybean lines, and the opposite results were detected in RNAi lines compared with those in EV lines. However, NAP suppressed drought-induced transcripts of all genes, especially *GmWRKY46* and *GmbZIP1* (Figure 8E and Figure 8F).

Transcriptional activation assays in *Arabidopsis* protoplasts

The promoters of 15 stress-responsive genes were analyzed by PLACE program (http://www.dna.affrc.go.jp/PLACE/signalscan.html), which showed that one to six CCAAT elements were found (Table S2). Furthermore, two stress-related genes (*GmDREB2* and *GmbZIP1*) were selected to explore whether GmNFYA5 could bind the promoters in vivo by an *Arabidopsis* protoplast transient expression system. Recombinant pGreenII 0800:*GmDREB2p/GmbZIP1p* vector was co-transformed into *Arabidopsis* protoplasts with an empty p16318GFP vector or a p16318GFP:*GmNFYA5* vector (Figure 9A). These assays showed that the protoplasts expressing GFP-GmNFYA5 exhibited significantly higher expression level of the LUC reporter gene compared with the GFP (Figure 9B and Figure 9C).

Discussion

Compared with other abiotic stresses, drought is more widespread and damaging [3]. Multiple transcriptional cascades mediate gene regulation with the treatment of drought [31, 32]. Transcription factor genes induced in these cascades in turn regulate the related downstream genes to resist drought stress. Our results shows that most of

NF-YA members respond to drought stress in soybean (Figure 1A), which are consistent with the previous observations [33]; among all the NF-YA genes, the transcript level of *GmNFYA5* is the highest one with the treatment of drought. Tissue specific expression analysis shows that transcript abundance of *GmNFYA5* in roots is higher compared with that in other tissues except for leaves (Figure 2A), which implies that *GmNFYA5* is connected with drought resistance. Likewise, several genes conferring drought tolerance to transgenic plants maintained the highest transcript abundance in roots under normal condition [25, 28, 34].

In addition to being induced by drought stress, *GmNFYA5* is regulated by ABA (Figure 1B). The NAP treatment suppresses drought-induced transcript of *GmNFYA5* (Figure 1C), which shows that transcript of *GmNFYA5* in response to drought depends on cross-talk of ABA signaling. It has been well documented that altered transcripts of NF-Y genes can affect the phenotypes and expression of stress-related genes via ABA-dependent pathway [25, 28, 30, 35]. In this study, overexpression of *GmNFYA5* confers drought tolerance to the transgenic *Arabidopsis* plants (Figure 5), and increased sensitivity to ABA based on germination rate and root length compared with WT plants (Figure 3). Meanwhile, the concentration of ABA and *NCED3* transcript in transgenic *Arabidopsis* are significantly higher compared with those in WT plants under drought stress (Figure 5G and Figure 6D). The observations show that overexpression of *GmNFYA5* increases the expression level of *NCED3* to enhance ABA biosynthesis, which increases ABA sensitivity in transgenic *Arabidopsis* plants. It was reported that overexpression of *GmNFYA3* [30], *AtNFYA5* [25], *Cdt-NFYC1* [28] and *GmTGA17* [36] confers increased ABA sensitivity and drought tolerance to transgenic lines respectively. Improved drought resistance accompanies high sensitivity to the treatment of ABA [34, 37]. As an important signaling intermediate, ABA controls the expression of many stress-induced genes [38, 39]. Additionally, Increased ABA concentration in transgenic *Arabidopsis* plants (Figure 5G) is likely to be associated with higher transcript levels of stress-related genes in transgenic *Arabidopsis* plants compared with WT plants.

ABA related marker genes, such as *ABI2*, *ABI3*, *LEA3*, *RD29A* and *P5CS1*,

maintain higher transcript levels in transgenic *Arabidopsis* plants compared with WT plants under normal and drought conditions (Figure 6D-I). In addition, *GmbZIP1* and *GmWRKY46*, ABA-dependent genes in soybean, are higher in transcript levels of OE plants compared with those of EV plants, and the opposite results are observed in RNAi plants under normal and drought conditions (Figure 8E and Figure 8F). NAP suppresses drought-induced expression level of these genes seriously (Figure 6 and Figure 8), which were certified to be positive regulators of drought tolerance [40-45]. The results show that *GmNFYA5* confers drought tolerance to transgenic *Arabidopsis* and soybean plants via ABA-dependent way. Additionally, the transcript levels of *DREB1A*, *DREB2A* and *DREB2B* in transgenic *Arabidopsis* plants are higher than those of WT plants (Figure 6A-C). *GmNFYA5* functions in positive regulation of ABA unrelated marker genes (*GmDREB1*, *GmDREB2* and *GmDREB3*) in soybean (Figure 8A-C). NAP affects drought-induced transcripts of these genes slightly (Figure 6 and Figure 8), Up-regulation of which improves drought tolerance through ABA-independent pathway [46-49]. Moreover, one to six CCAAT *cis*-acting elements are found in the promoter of all the marker genes used in this study (Table S2). Transcriptional activation assays shows that promoters of *GmDREB2* and *GmbZIP1* are binded by GmNFYA5 to enhance the expression level of LUC reporter gene in vivo (Figure 9B and Figure 9C). The results give further insights into the regulation of drought tolerance by *GmNFYA5* through ABA-dependent and ABA-independent pathways in *Arabidopsis* and soybean.

Conclusions

Transgenic soybean and *Arabidopsis* plants overexpressing *GmNFYA5* exhibit enhanced drought tolerance depended on the phenotypic indexes and transcripts of drought-related genes. *GmNFYA5* is likely to be a positive gene which can increase drought resistance and has potential application value in molecular breeding of soybean.

Methods

Plant growth and treatments

Soybean (*Glycine max* L. Merr.) cultivar Williams 82 was grown in plastic pots (15 cm in diameter and 20 cm in depth) containing a mixture of peat and vermiculite (1:1, v/v), 20-day-old seedlings were used to evaluate its expression patterns. For drought treatment, the whole plant was pulled up and washed cleanly, placed in a laminar flow hood for gradual drought for 12 h [28]. For ABA treatment, roots of soybean seedlings were subjected to 100 μM ABA for 12 h. During the two treatments, the leaves of soybean were collected at 0, 1, 2, 4, 8 and 12 h for isolation of RNA. To understand whether ABA was involved in drought-induced transcription of *GmNFYA5*, detached leaves were placed in H_2O for 1 h to eliminate the influence of the wound stress, and then treated with distilled water or 1 mM naproxen (NAP), which can inhibit the synthesis of ABA, followed by drought treatment for 2 h. The leaves floated in H_2O were normal control.

Transgenic *Arabidopsis* lines and ecotype Columbia-0 (WT) seedling were used in this study. Seeds were surface-sterilized with 70% ethanol and washed with sterile water for three times, followed by being sterilized with 1% sodium hypochlorite for 15 minutes and washed with sterile water for three times. Then the sterilized seeds were sown on half-strength Murashige and Skoog medium (1/2 MS, 2% sucrose, 0.8% agar). After stratified at 4°C for 2 days in darkness, they were placed in a tissue culture room at 22°C with a 16-h photoperiod. For drought treatment, 3-week-old seedlings which were transferred to plastic pots (8 cm in length, width and depth) containing a mixture of peat and vermiculite (1:1, v/v) for 7 days were shut off water supply until they became wilting. Three biological replicates were performed for each line.

To investigate the transcript of marker genes under conditions of drought and drought pretreat with NAP in transgenic *Arabidopsis* plants, 3-week-old seedlings were placed into H_2O or 1 mM NAP solution, and then transferred to a laminar flow hood for 2 h as drought treatment. The seedlings floated in H_2O were normal control.

Leaves were sampled for isolation of RNA. Three biological replicates were performed for each line.

The seeds of soybean were provided from Dr. Li-Juan Qiu of the institute of Crop Science, Chinese Academy of Agricultural Sciences. The seeds of *Arabidopsis* were bought from ABRC (https://abrc.osu.edu/researchers).

Germination rate and root growth assay

The sterilized seeds were sown on 1/2 MS medium with 8-10% PEG 6000 (PEG) and 0.5-0.8 μM ABA respectively and placed in a tissue culture room at 22°C after stratified at 4°C for 2 d in darkness. The germination rates were recorded every 12 hours until the seed germination was completed. To investigate the root elongation of the transgenic *Arabidopsis* lines, 3-day-old seedlings were exposed to 1/2 MS medium with 10-12% PEG and 0.5-1 μM ABA. A week later, the length of roots were measured in all plants. All the measurements above were replicated three times biologically.

Isolation of *GmNFYA5*

Soybean cultivar Williams 82 was used to isolate the total RNA as described previously [34]. The full-length cDNA of *GmNFYA5* was obtained by PCR with KOD-Plus DNA polymerase (TOYOBO, Japan) with the following primers: forward 5'-GTAAGTGCGACTCTAAGCAAGCCT-3' and reverse 5'-TAATGTAAATGAGCCAAGGATGACT-3'. The amplified products were purified and cloned into the pEASY-Blunt vector (TransGen, China) for sequencing.

Analysis of transcript levels

Quantitative real-time PCR (qRT-PCR) was performed with TransStart Top Green qPCR SuperMix (TransGen, China) according to the manufacturer's instructions on an Applied Biosystems 7500 real-time PCR system. Three biological replicates were done for each experiment in a total volume of 20 μl. Gene-specific primers used for qRT-PCR assays were listed in Table S1.

Subcellular localization

The coding sequence (CDS) of *GmNFYA5* without the termination codon was fused in frame to the N-terminus of GFP in the vector p16318GFP, and ligated with *Bam*HI

site to generate a p16318GFP:*GmNFYA5* fusion construct under the control of CaMV35S promoter using a primer set 5'-TATCTCTAGAGGATCCATGAAGAACTTATGTGAG-3' and 5'-TGCTCACCATGGATCCCATAAGGACTGATAGACG-3'. The CDS of *GmNFYA3* encoding a nuclear-localized protein [30] was cloned into the *Eco*RI site of a vector named pLVX-IRES-mCherry using a primer set 5'-TCTATTTCCGGTGAATTCATGCAAACTGTTTATCTT-3' and 5'-ACTAGTCTCGAGGAATTCAACTTTAAGGTTGCAGCA-3'. The GmNFYA3:mCherry fusion protein was used for the marker of the nucleus, *Arabidopsis* protoplasts were prepared as described [50]. Transfected protoplasts were incubated in darkness at 22°C for 16-18 h, GFP fluorescence signals were observed with a confocal laser scanning microscope named LSIM 700 (Zeiss) and repeated three times biologically [51].

Transcriptional activation assays

The promoters of *GmDREB2* and *GmbZIP1* were cloned into the LUC reporter vector pGreen II 0800 containing Renilla luciferase (REN) gene used as an internal control driven by the CaMV 35S promoter. The effector and reporter plasmids were extracted and introduced into the *Arabidopsis* protoplasts using PEG4000-mediated transformation. The assays were performed as described previously [6].

Generation rate of transgenic *Arabidopsis* lines

The CDS of *GmNFYA5* was cloned into the *Nco*I site of a vector named pCAMBIA1302 and driven by CaMV35S promoter using a primer set 5'-GGGACTCTTGACCATGATGAAGAACTTATGTGAG-3' and 5'-TCAGATCTACCATGGCCATAAGGACTGATAGACG-3'. The pCAMBIA1302:*GmNFYA5* construct was introduced into *Agrobacterium tumefaciens* GV3101 and infected *Arabidopsis* using the floral dip method. The positive transgenic *Arabidopsis* lines were screened by hygromycin (Roche, Germany) to select the T_1 and T_2 plants to harvest homozygous transgenic seeds.

Promoter:GUS analysis

The promoter of *GmNFYA5* was amplified from the DNA of soybean cultivar

Williams 82 with the forward primer 5'-AAGAGGAACACAGAAGTCTATGAGT-3' and reverse primer 5'-GCACATCAGATTCAGAGGAAGTCCC-3'. The products were introduced into a reconstructive pCAMBIA1305 vector (GFP coding region was replaced by GUS coding region) incorporating *Eco*RI and *Nco*I sites with the forward primer 5'-CCATGATTACGAATTCAAGAGGAACACAGAAGTC-3' and reverse primer 5'-CTCAGATCTACCATGGCTCACATAAGTTCTTCAT-3'. The construct was introduced into *A. tumefaciens* GV3101 and transferred into *Arabidopsis* Col-0 plants by the floral dip method. The positive transgenic *Arabidopsis* lines were screened by hygromycin to harvest homozygous seeds.

A. rhizogenes-mediated transformation of soybean hairy roots

The CDS of *GmNFYA5* was inserted into the pCAMBIA3301 vector incorporating *Nco*I and *Bst*EII sites with the following primers: forward 5'-GGACTCTTGACCATGATGAAGAACTTATGTGAG-3' and reverse 5'-ATTCGAGCTGGTCACCCATAAGGACTGATAGACG-3'. Meanwhile a 635-bp synthetic RNAi hairpin fragment (Figure S2) was introduced into the pCAMBIA3301 vector and ligated with the same sites as above. The pCAMBIA3301:*GmNFYA5*, pCAMBIA3301 empty vector and pCAMBIA3301:RNAi-*GmNFYA5* construct were introduced into *A. rhizogenes* K599 and used to infect the hypocotyls of 5-day-old soybean, and then hairy roots were induced for two weeks [52]. The positive hairy roots were screened using QuickStix kit for PAT/bar (EnviroLogix, America) and qRT-PCR. Transgenic soybean lines having positive hairy roots were named OE-*GmNFYA5* (OE), empty vector (EV) and RNAi-*GmN*FYA5 (RNAi) respectively and transferred to plastic pots (11 cm in depth, 13.5 cm in diameter) containing a mixture of peat and vermiculite (1:1, v/v) to grow for 7 days, followed by shutting off water supply until they became wilting. To analyze the transcription of drought related genes under conditions of drought and drought pretreated with NAP in transgenic soybean plants, positive hairy roots were placed into H_2O or 1 mM NAP solution, and then transferred to a laminar flow hood for 2 h as drought treatment. The roots floated in H_2O were normal control. Roots were sampled for isolation of RNA. Three biological replicates were performed for each line.

Stomatal aperture analysis

The epidermises of leaves were treated for 3 h in stomatal opening buffer (5 mM MES, 10 mM KCl, 50 mM $CaCl_2$, pH 5.6), following by being exposed to 10 μM ABA, which was described previously [53]. Stomatal apertures of stomatal complexes with no mesophyll cells surrounding them were analyzed and measured thereafter. Three biological replicates were done for each line.

Measurement of water loss, malondialdehyde (MDA) and ABA content

Leaves of 3-week-old *Arabidopsis* lines were detached, and the weight was measured every 30 minutes under the same condition. Each line was determined in three replicates. The percentage of initial fresh weight at 9 time points was used to represent water loss of transgenic *Arabidopsis* and WT plants. MDA contents were measured and calculated as described previously [51, 54]. All the measurements above were replicated three times biologically.

To measure the concentrations of ABA in transgenic *Arabidopsis* and WT plants, ABA ELISA assay kit (Jiancheng, China) for plant was used as described previously [55]. All the measurements were replicated three times biologically.

Measurement of relative water content (RWC) and ion leakage

RWC and ion leakage were determined when plants became wilting as described previously [56, 57]. All the measurements were replicated three times biologically.

Statistical analysis

All the data were subjected to analysis with Student's t test. All the measurements were replicated three times biologically to calculate the standard errors.

Abbreviations

GFP, Green fluorescent protein; NAP, naproxen; RWC, relative water content; MDA, malondialdehyde; ABA, abscisic acid

Declarations

Ethics approval and consent to participate

This research is not applicable to the ethics approval and consent to participate.

Consent for publication

This research is not applicable to the consent for publication.

Availability of data and material

The data in this research are available in https://www.arabidopsis.org/, https://phytozome.jgi.doe.gov/pz/portal.html and https://www.ncbi.nlm.nih.gov/.

Competing interests

The authors declare no competing financial interests.

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Authors' contributions

ZSX and JHZ coordinated the project, conceived and designed experiments, and edited the manuscript; XJM performed experiments and wrote the first draft; TFY, XHL, WJZ, XYC and JM conducted the bioinformatic work and performed experiments; JC, YBZ and MC provided analytical tools and managed reagents; YZM coordinated the project; XJM contributed with valuable discussions. All authors have read and approved the final manuscript.

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Figure legends

Figure 1 *GmNFYA5* expression as affected by drought stress and ABA. (A) Transcripts of 21 NF-YA genes were analyzed using qRT-PCR in soybean under drought stress. The expression levels were normalized to that of *GmCYP2*. *GmNFYA5* transcript at 0 h was set at 1.0, and SD for three biological replicates is represented by error bars. **(B)** Transcript of *GmNFYA5* was analyzed in response to ABA treatment in soybean. The expression levels were normalized to that of *GmCYP2*. *GmNFYA5* transcript at 0 h was set at 1.0, and SD for three biological replicates is represented by error bars. **(C)** qRT-PCR of *GmNFYA5* transcript in soybean plants in response to drought stress with the treatment of 1 mM NAP. Control, drought and drought pretreated with NAP are indicated by CTR, D and D + NAP respectively. The level of *GmNFYA5* transcript under control condition was set at 1.0, and the internal control was *GmCYP2*. SD for three biological replicates is represented by error bars. Significant differences at $0.01 < P < 0.05$ and $P < 0.01$ are indicated by * and ** above the columns respectively.

Figure 2 Tissue specific expression analysis and subcellular localization of *GmNFYA5*. (A) Expression abundance of the *GmNFYA5* gene in soybean tissues at seedling and flowering stages. The tissues of soybean under normal condition were used to extract total RNA. The relative transcript level of *GmNFYA5* in soybean tissues is indicated by the *vertical* column. *GmNFYA5* transcript in roots was set at 1.0, and the internal control was *GmCYP2*. SD for three biological replicates is represented by error bars. Significant differences at $0.01 < P < 0.05$ and $P < 0.01$ are indicated by * and ** above the columns respectively. **(B)** Expression pattern of *GmNFYA5* promoter:GUS in various tissues of transgenic *Arabidopsis* plants. *a* 5-day-old seedling, *b* rosette leaf, *c* cauline leaf, *d* root, *e* flower , *f* silique. Each experiment was repeated three times biologically. **(C)** Subcellular localization of GmNFYA5. The fluorescence of GFP-GmNFYA5 and mCherry-GmNFYA3 fusion proteins in transformed cells was localized exclusively to the nucleus collectively. Scale bar = 10 μm. Three biological replicates were performed for each experiment.

683 **Figure 3 Germination rate and root growth of WT and transgenic *Arabidopsis***
684 **plants with ABA treatment. (A)** Seed germination on 1/2 MS agar plates with 0, 0.5
685 and 0.8 μM ABA. SD for three biological replicates (n = 64) is represented by error
686 bars. **(B) and (C)** The root growth of WT and transgenic *Arabidopsis* plants on media
687 with/without ABA. 3-day-old seedlings from 1/2 MS medium were transferred to the
688 media containing 0, 0.5 and 1 μM ABA, 7 days later the photographs were taken.
689 Scale bar = 2 cm. Each experiment was repeated three times biologically and similar
690 results were obtained. Each measurement represents the average root length of thirty
691 seedlings ± SD. Significant differences at $0.01 < P < 0.05$ and $P < 0.01$ are indicated
692 by * and ** above the columns respectively.
693 **Figure 4 Germination rate and root growth of WT and transgenic *Arabidopsis***
694 **plants with PEG treatment. (A)** Seed germination on 1/2 MS agar plates with 8 and
695 10% PEG. SD for three biological replicates (n = 64) is represented by error bars. **(B)**
696 **and (C)** The root growth of WT and transgenic *Arabidopsis* plants on media
697 with/without PEG. 3-day-old seedlings from 1/2 MS medium were transferred to the
698 media containing 0, 10 and 12% PEG, 7 days later, the photographs were taken. Scale
699 bar = 2 cm. Each experiment was repeated three times biologically and similar results
700 were obtained. Each measurement represents the average root length of thirty
701 seedlings ± SD. Significant differences at $0.01 < P < 0.05$ and $P < 0.01$ are indicated
702 by * and ** above the columns respectively.
703 **Figure 5 Improved drought tolerance and stomatal aperture analysis in**
704 **transgenic *Arabidopsis* plants. (A)** Assessment of drought tolerance in transgenic
705 *Arabidopsis* plants. 3-week-old plants were grown without water supply for 14 days,
706 followed by re-watering for 7 days. Each experiment was repeated three times (n = 64)
707 biologically. Drought resistance of transgenic *Arabidopsis* plants was assayed by the
708 capability to resume growth when returned to normal conditions after drought stress.
709 **(B)** *GmNFYA5* transcript was detected in three transgenic *Arabidopsis* lines. The
710 expression levels were normalized to that of *Tub8*. Transcript of *GmNFYA5* in WT
711 plants was set at 1.0, and SD for three biological replicates is represented by error
712 bars. **(C-G)** Measurements of survival rate, water loss, ion leakage, RWC and

concentrations of ABA in transgenic *Arabidopsis* and WT plants. Three biological replicates (n = 64) were performed for each line. **(H-I)** The stomatal aperture in transgenic *Arabidopsis* and WT plants with normal and 10 μM ABA treatments, the width/length of the stomatal aperture was measured using the ruler tool of Adobe Photoshop CS5. Scale bar = 5 μm. SD for three biological replicates (n = 40) is represented by error bars. Significant differences at $0.01 < P < 0.05$ and $P < 0.01$ are indicated by * and ** above the columns respectively.

Figure 6 Relative transcript levels of *DREB1A*, *DREB2A*, *DREB2B*, *ABI2*, *ABI3*, *NCED3*, *LEA3*, *RD29A* and *P5CS1* in transgenic *Arabidopsis* and WT plants under three conditions. The relative transcript level is indicated by the *vertical* column and normalized to that of *Tub8*. Transcripts of stress-related genes in WT plants under normal condition was set at 1.0, SD for three biological replicates is represented by error bars. Control, drought and drought pretreated with NAP are indicated by CTR, D and D + NAP respectively. Significant differences compared with the WT plants at $0.01 < P < 0.05$ and $P < 0.01$ are indicated by * and ** above the columns respectively.

Figure 7 Improved drought tolerance in transgenic soybean plants.

(A-C) Assessment of drought tolerance in transgenic soybean plants. Transgenic soybean plants with positive hairy roots were transferred to plastic pots containing a mixture of peat and vermiculite (1:1, v/v) to grow for 7 days, and then grown without water supply for 16 days, followed by re-watering for 3 days. Each experiment was repeated three biological replicates (n = 64). Drought resistance of transgenic soybean plants was assayed by the capability to resume growth when returned to normal conditions after drought stress. Scale bar = 5 cm. **(D)** Relative transcript of *GmNFYA5* was detected in three transgenic soybean lines. Transcript of *GmNFYA5* in EV plants was set at 1.0, and the expression levels were normalized to that of *GmCYP2*. SD for three biological replicates is represented by error bars. **(E-H)** Measurements of MDA, RWC, ion leakage, survival rate in transgenic soybean plants. Data represent mean SD for three biological replicates (n = 64). Significant differences at $0.01 < P < 0.05$ and $P < 0.01$ are indicated by * and ** above the columns respectively.

Figure 8 Relative transcript levels of *GmDREB1*, *GmDREB2*, *GmDREB3*, *GmNCED2*, *GmWRKY46* and *GmbZIP1* in transgenic soybean and EV plants under three conditions. The relative transcript level is indicated by the *vertical* column and normalized to that of *GmCYP2*. Transcripts of stress-related genes in EV plants under normal condition was set at 1.0, SD for three biological replicates is represented by error bars. Control, drought and drought pretreated with NAP are indicated by CTR, D and D + NAP respectively. Significant differences compared with the EV plants at $0.01 < P < 0.05$ and $P < 0.01$ are indicated by * and ** above the columns respectively.

Figure 9 GmNFYA5 increase the activity of LUC reporter gene by binding the promoters of *GmDREB2* and *GmbZIP1*. (A) The structures of effector and reporters. **(B) and (C)** The ratio of the LUC to REN indicates the activity of GmNFYA5 on the transcript of the *GmDREB2* and *GmbZIP1* promoters. SD for three biological replicates ($n = 10$) is represented by error bars. Significant differences at $0.01 < P < 0.05$ and $P < 0.01$ are indicated by * and ** above the columns respectively.

Supplemental data

The following materials are available in the online version of this article.

Figure S1 Sequence alignment of the conserved domains of GmNFYA5 and members of NF-YA family in *Arabidopsis*. (A) Sequence alignment of the conserved domains in GmNFYA5 and 10 members of NF-YA family in *Arabidopsis*, two subdomains and linker are underlined. Asterisks indicate critical amino acids. **(B)** Phylogenetic analysis of *GmNFYA5* with 10 members of NF-YA family in *Arabidopsis*. An unrooted neighbor joining tree was constructed using MEGA 7.0.

Figure S2 The sequence of RNAi-*GmNFYA5* which was inserted into the pCAMBIA3301 vector incorporating *Nco*I and *Bst*EII sites to generate the RNAi soybean line. The hairpin structure is composed of three sequences: the positive sequence of RNAi-*GmNFYA5* in blue color, the reverse complementary sequence in green color and an intron of *GmNFYA5* in purple color.

Table S1 List of primers used in this study.

Table S2 Promoter sequence analysis of genes up-regulated by *GmNFYA5* in transgenic *Arabidopsis* and soybean lines.

Figures

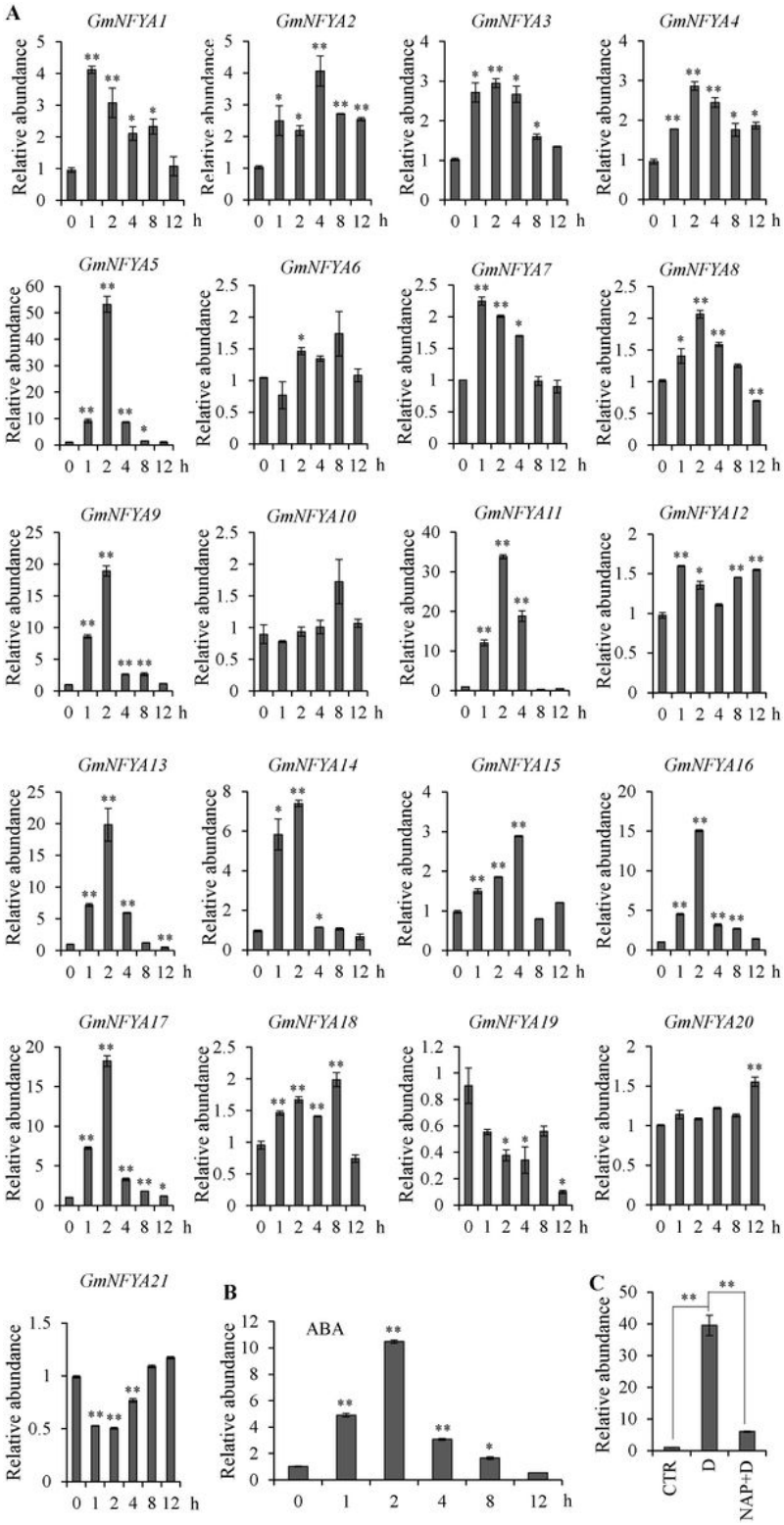

Figure 1

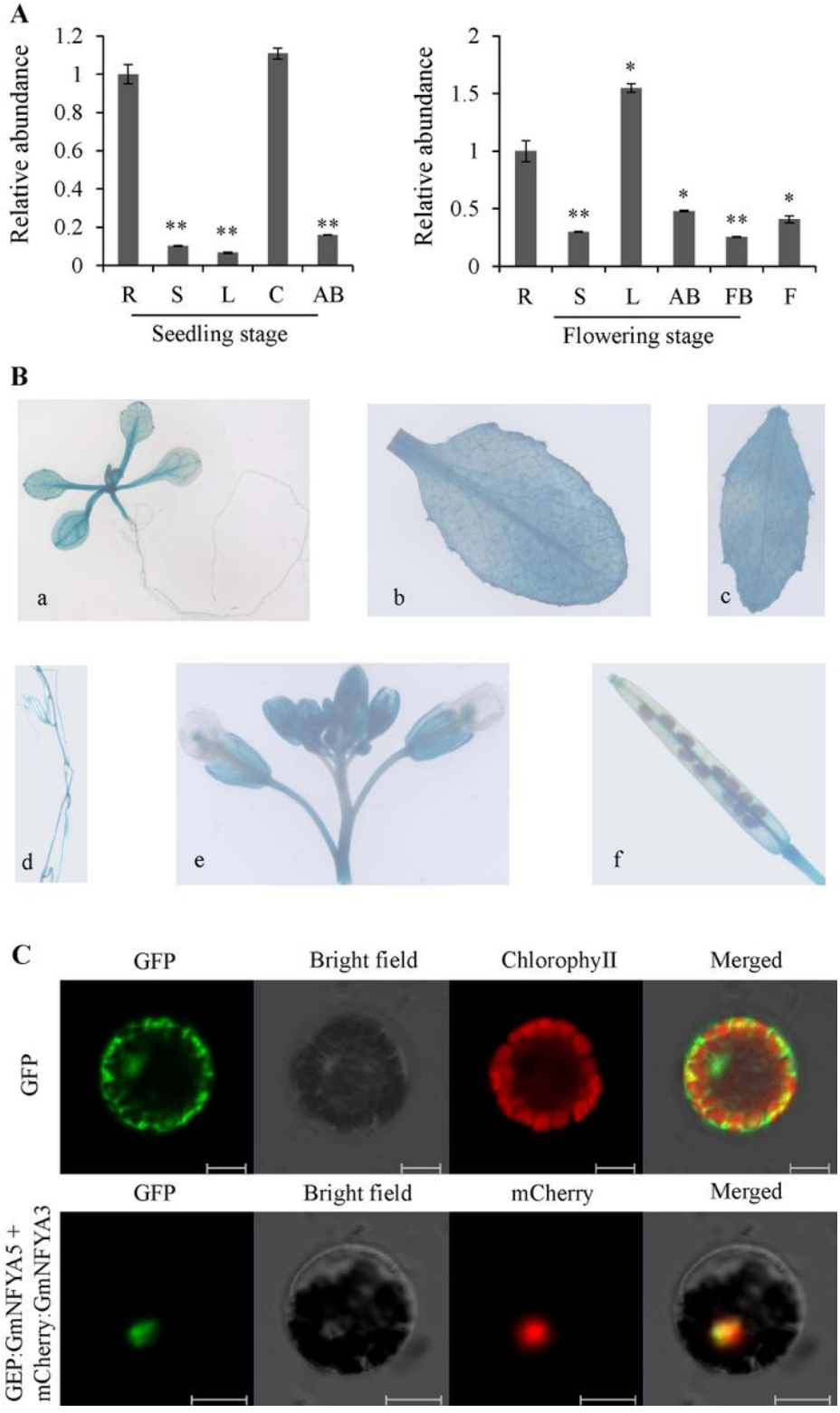
A
Relative abundance
1.2
1
0.8
0.6
0.4
0.2
0
**
**
**
R
S
L
C
AB
Seedling stage
Relative abundance
2
1.5
1
0.5
0
*
**
*
**
*
R
S
L
AB
FB
F
Flowering stage
B
a
b
c
d
e
f
C
GFP
Bright field
ChlorophyII
Merged
GFP
GFP
Bright field
mCherry
Merged
GEP:GmNFYA5 +
mCherry:GmNFYA3

Figure 2

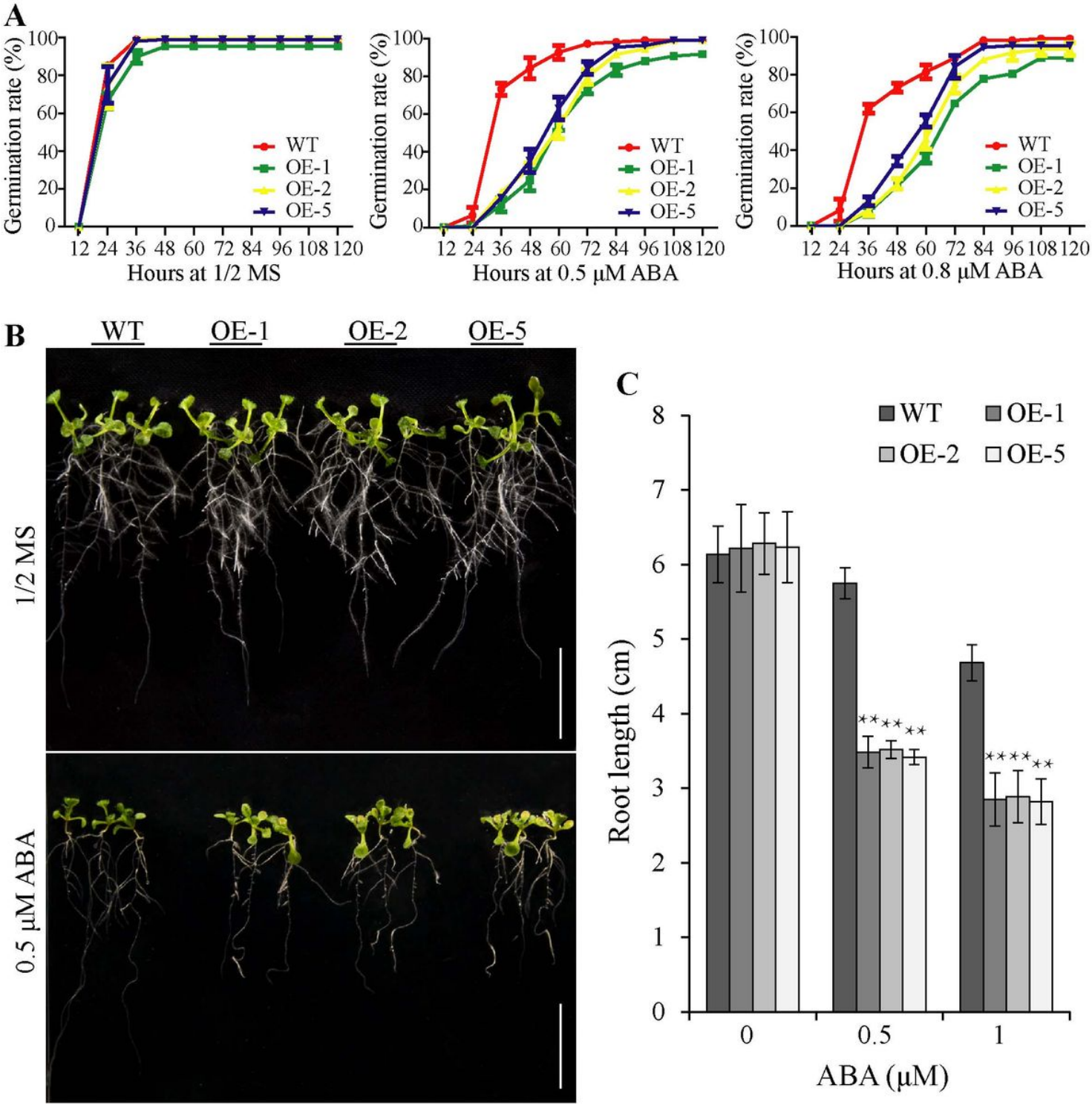
A
Germination rate (%)
WT
OE-1
OE-2
OE-5
Hours at 1/2 MS
Hours at 0.5 μM ABA
Hours at 0.8 μM ABA
B
WT
OE-1
OE-2
OE-5
1/2 MS
0.5 μM ABA
C
Root length (cm)
ABA (μM)

Figure 3

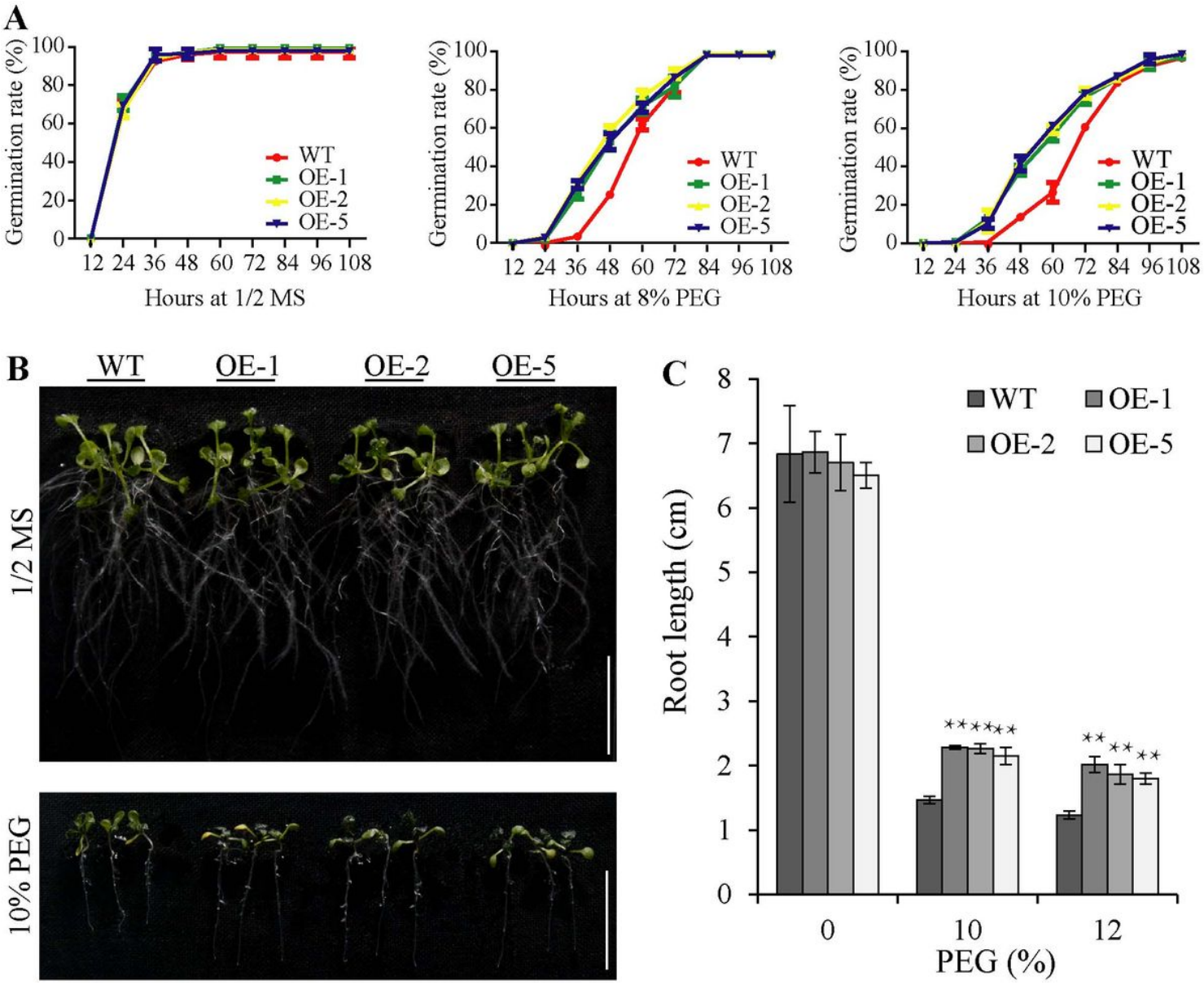
A
Germination rate (%)
100
80
60
40
20
0
12 24 36 48 60 72 84 96 108
Hours at 1/2 MS
WT
OE-1
OE-2
OE-5
Germination rate (%)
Hours at 8% PEG
Germination rate (%)
Hours at 10% PEG
B
WT
OE-1
OE-2
OE-5
1/2 MS
10% PEG
C
Root length (cm)
8
7
6
5
4
3
2
1
0
0
10
12
PEG (%)

Figure 4

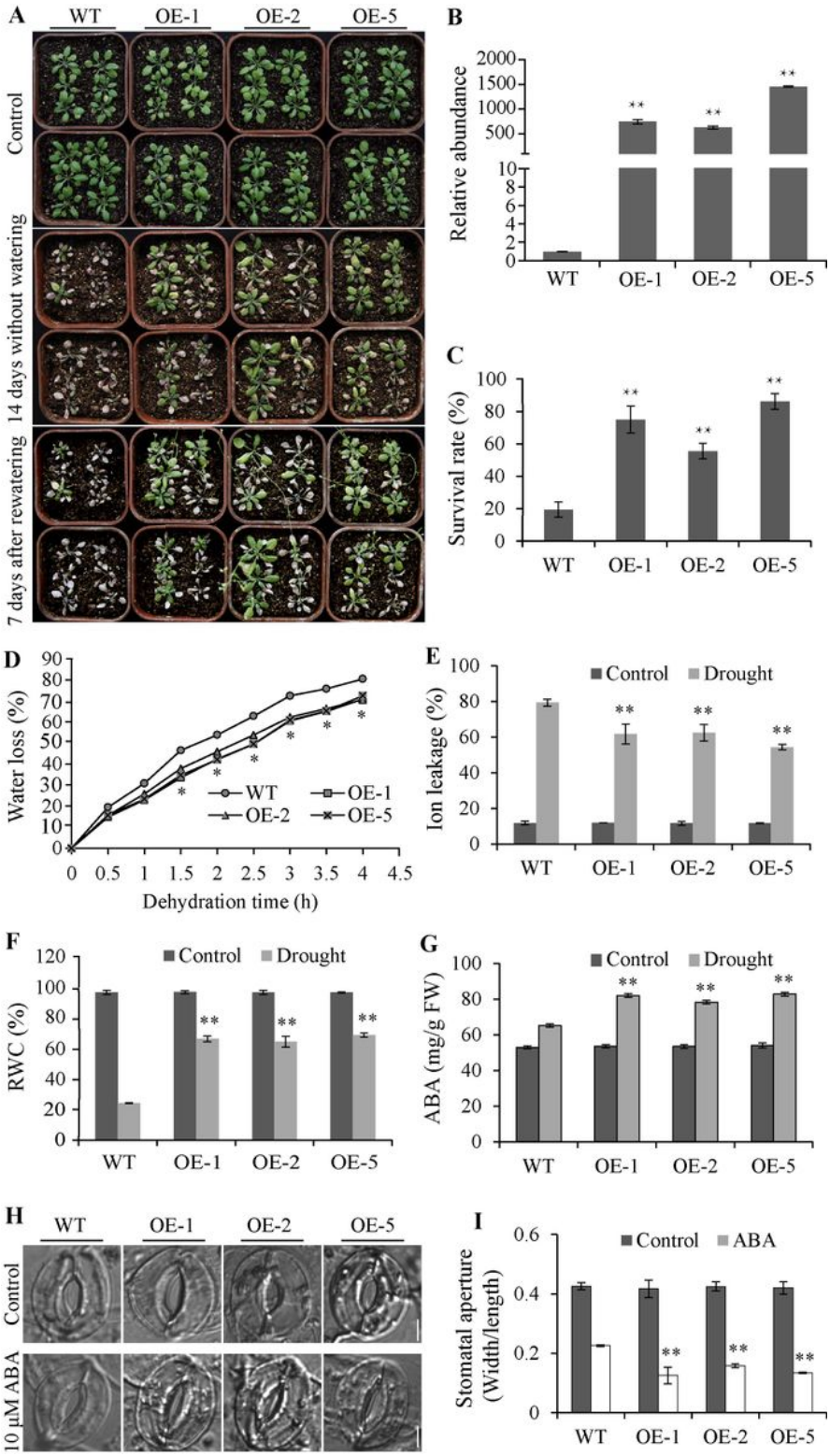
A
WT
OE-1
OE-2
OE-5
Control
14 days without watering
7 days after rewatering
B
Relative abundance
2000
1500
1000
500
10
8
6
4
2
0
**
WT
OE-1
OE-2
OE-5
C
Survival rate (%)
100
80
60
40
20
0
**
WT
OE-1
OE-2
OE-5
D
Water loss (%)
90
80
70
60
50
40
30
20
10
0
WT
OE-1
OE-2
OE-5
0 0.5 1 1.5 2 2.5 3 3.5 4 4.5
Dehydration time (h)
E
Ion leakage (%)
Control
Drought
100
80
60
40
20
0
**
WT
OE-1
OE-2
OE-5
F
RWC (%)
Control
Drought
120
100
80
60
40
20
0
**
WT
OE-1
OE-2
OE-5
G
ABA (mg/g FW)
Control
Drought
100
80
60
40
20
0
**
WT
OE-1
OE-2
OE-5
H
WT
OE-1
OE-2
OE-5
Control
10 μM ABA
I
Stomatal aperture (Width/length)
Control
ABA
0.6
0.4
0.2
0
**
WT
OE-1
OE-2
OE-5

Figure 5

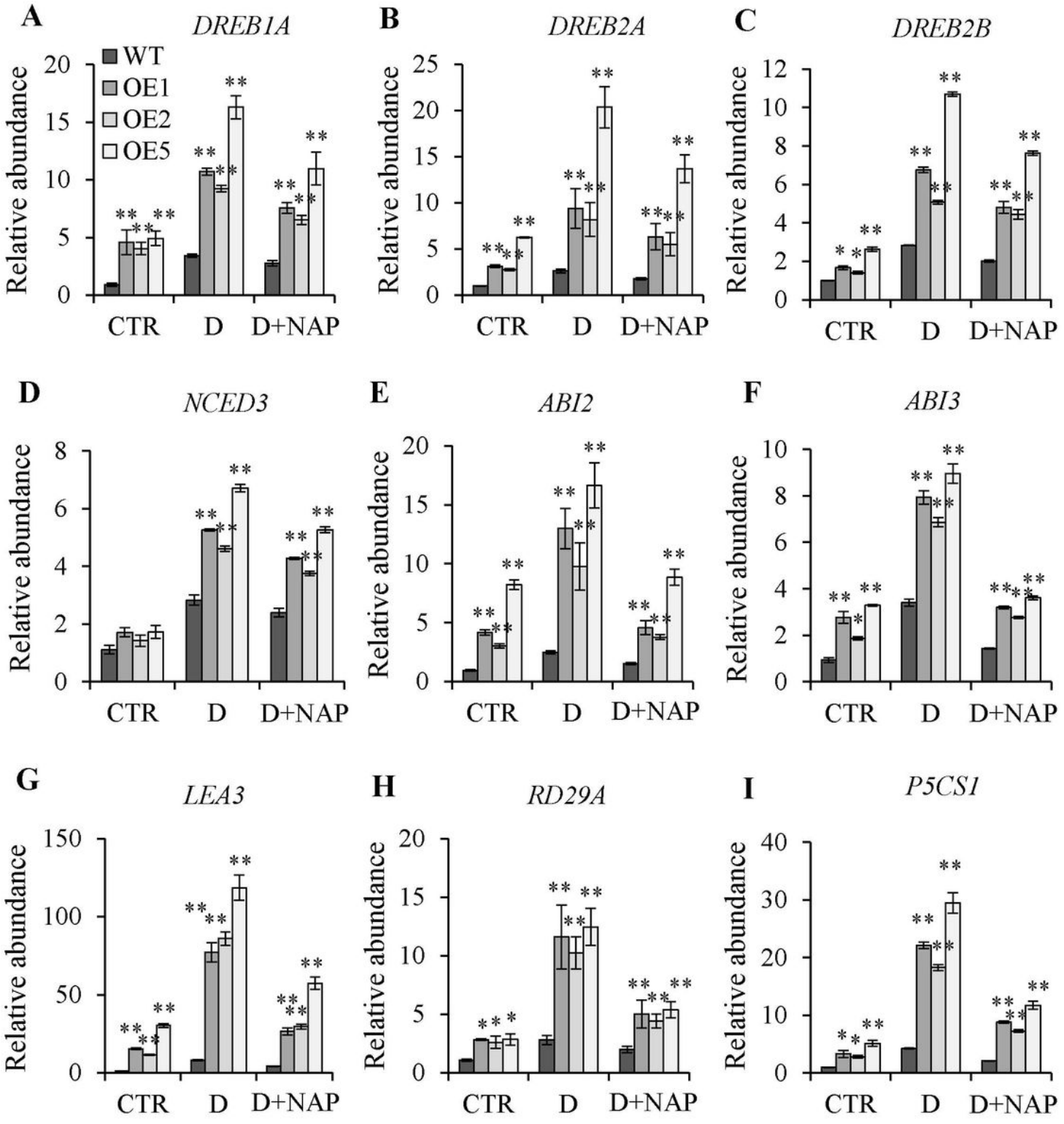
A
DREB1A
WT
OE1
OE2
OE5
Relative abundance
CTR
D
D+NAP
B
DREB2A
C
DREB2B
D
NCED3
E
ABI2
F
ABI3
G
LEA3
H
RD29A
I
P5CS1

Figure 6

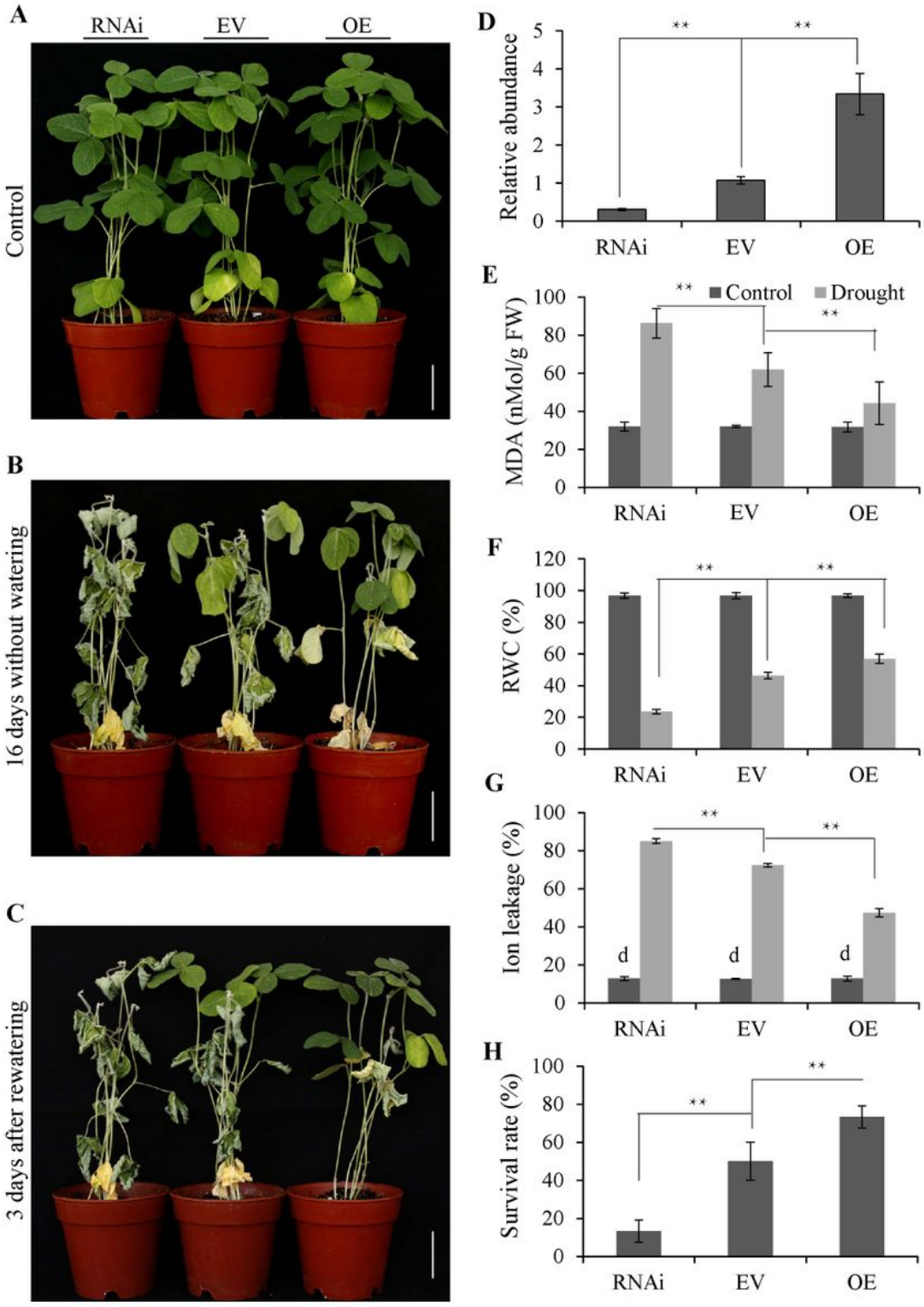
A
RNAi
EV
OE
Control
B
16 days without watering
C
3 days after rewatering
D
Relative abundance
RNAi
EV
OE
E
Control
Drought
MDA (nMol/g FW)
F
RWC (%)
G
Ion leakage (%)
d
H
Survival rate (%)

Figure 7

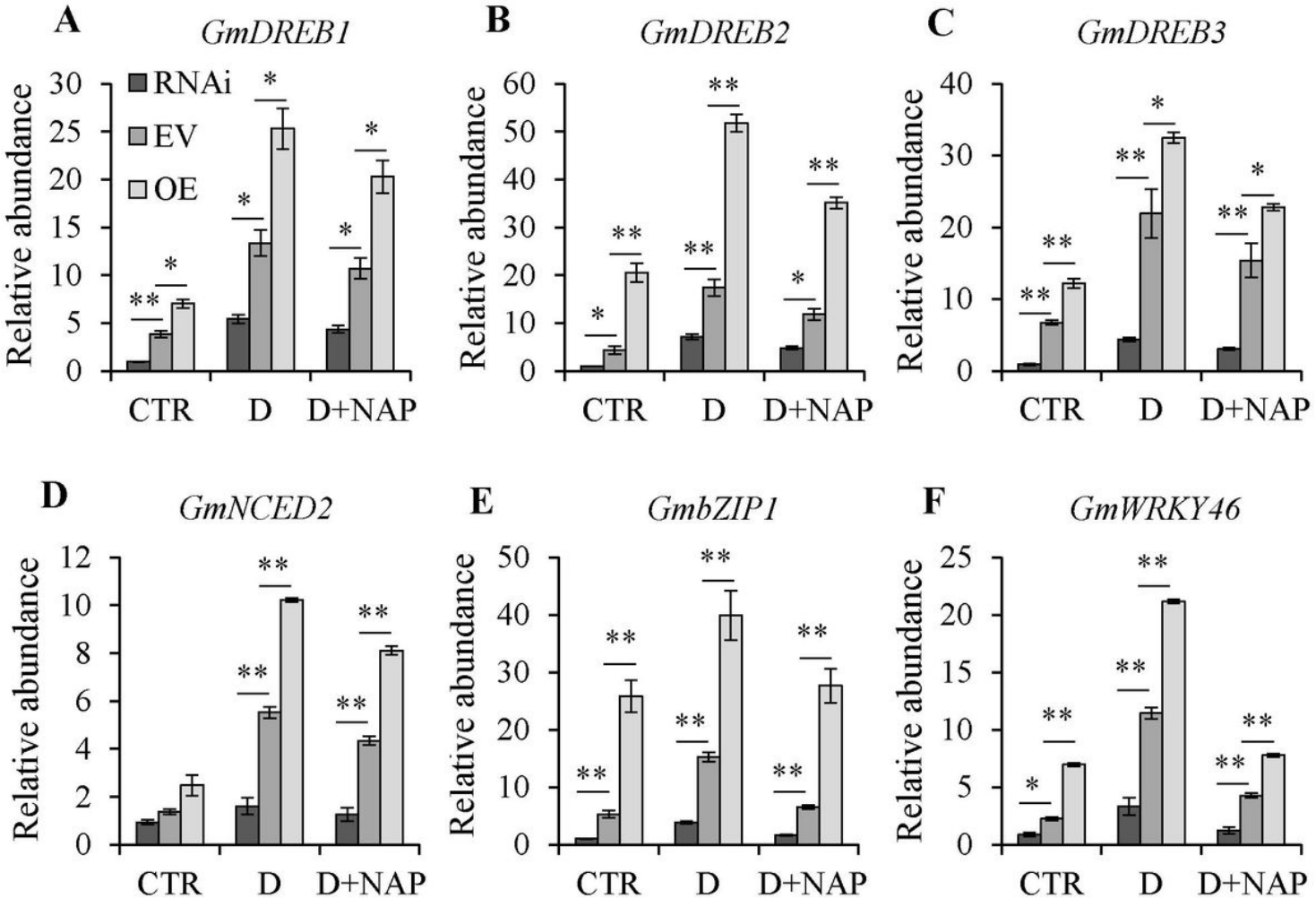

Figure 8

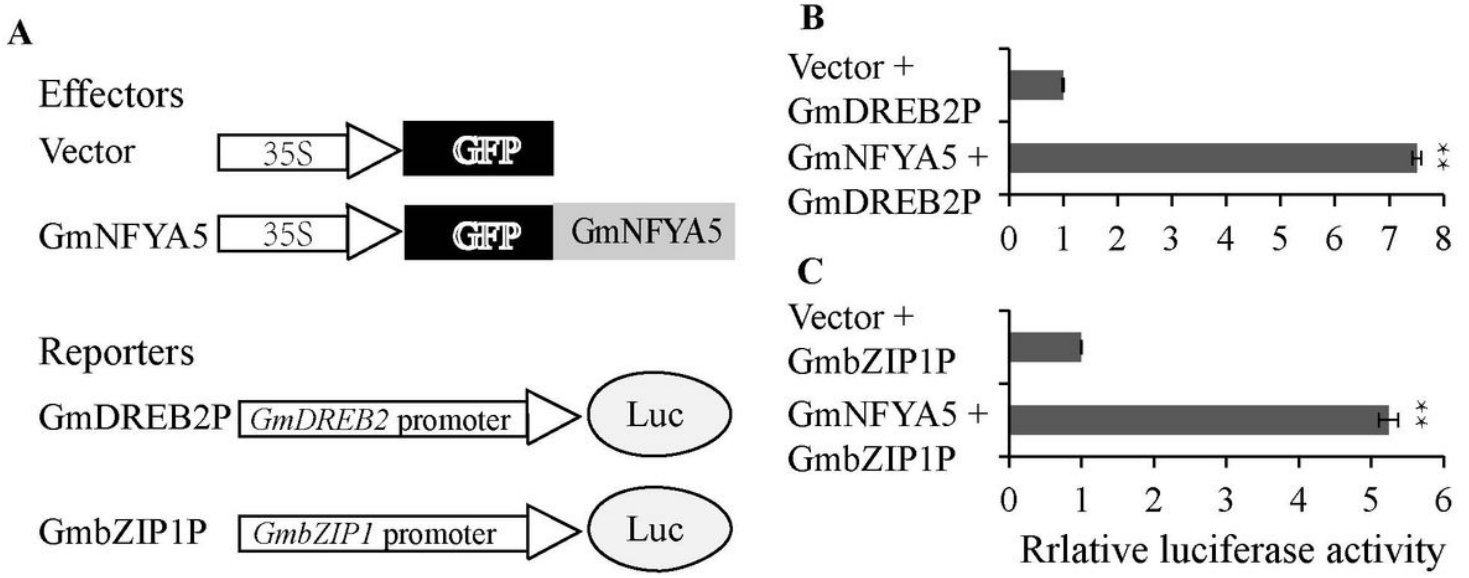

Figure 9

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- FigureS2.tif
- TableS1.xlsx
- TableS2.xlsx
- FigureS1.tif