Molecules and Cells

Confirmation of Drought Tolerance of Ectopically Expressed *AtABF3* Gene in Soybean

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Soybean transgenic plants with ectopically expressed *AtABF3* were produced by *Agrobacterium*-mediated transformation and investigated the effects of *AtABF3* expression on drought and salt tolerance. Stable *Agrobacterium*-mediated soybean transformation was carried based on the half-seed method (Paz et al. 2006). The integration of the transgene was confirmed from the genomic DNA of transformed soybean plants using PCR and the copy number of transgene was determined by Southern blotting using leaf samples from T_2 seedlings. In addition to genomic integration, the expression of the transgenes was analyzed by RT-PCR and most of the transgenic lines expressed the transgenes introduced. The chosen two transgenic lines (line #2 and #9) for further experiment showed the substantial drought stress tolerance by surviving even at the end of the 20-day of drought treatment. And the positive relationship between the levels of *AtABF3* gene expression and drought-tolerance was confirmed by qRT-PCR and drought tolerance test. The stronger drought tolerance of transgenic lines seemed to be resulted from physiological changes. Transgenic lines #2 and #9 showed ion leakage at a significantly lower level ($P < 0.01$) than non-transgenic (NT) control. In addition, the chlorophyll contents of the leaves of transgenic lines were significantly higher ($P < 0.01$). The results indicated that their enhanced drought tolerance was due to the prevention of cell membrane damage and maintenance of chlorophyll content. Water loss by transpiration also slowly proceeded in transgenic plants. In microscopic observation, higher stomata closure was confirmed in transgenic lines. Especially, line #9 had 56% of completely closed stomata whereas only 16% were completely open. In subsequent salt tolerance test, the apparently enhanced salt tolerance of transgenic lines was measured in ion leakage rate and chlorophyll contents. Finally, the agronomic characteristics of ectopically expressed *AtABF3* transgenic plants (T_2) compared to NT plants under regular watering (every 4 days) or low rate of watering condition (every 10 days) was investigated. When watered regularly, the plant height of drought-tolerant line (#9) was shorter than NT plants. However, under the drought condition, total seed weight of line #9 was significantly higher than in NT plants ($P < 0.01$). Moreover, the pods of NT plants showed severe withering, and most of the pods failed to set normal seeds. All the evidences in the study clearly suggested that overexpression of the *AtABF3* gene conferred drought and salt tolerance in major crop soybean, especially under the growth condition of low watering.

Keywords: *Agrobacterium*-mediated transformation, *AtABF3*, drought tolerance, soybean, stomatal closure

INTRODUCTION

Stress conditions, including drought, salinity, and cold, cause severe problems in the production of agricultural crops.

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Among abiotic stresses, drought stress is one of the most serious limiting factors for plant growth and crop yield. Shortage of water availability leads to a decisive yield losses in most environments (Sinclair et al., 2004; Zhang et al., 2010; Zhu, 2002). Recently, global warming has made a negative impact in agricultural regions. As a climate change limits the water resources for agricultural irrigation, the development of drought tolerant crops has become an important task worldwide. In order to improve the agricultural value under water stress, genetic transformation technology has been used to produce genetically modified (GM) crop varieties for commercial cultivation (Bruce et al., 2002; Li et al., 2017; Xoconostle-Cázares et al., 2010). Plants show a range of adaptations to environmental stresses and use various defense mechanisms to deal with drought stress, including drought escape, drought avoidance, and drought tolerance (Manavalan et al., 2009; Wang et al., 2016; Yang et al., 2011). Abscisic acid (ABA), an important phytohormone, plays an essential role in the adaptive response to abiotic stresses in higher plants during vegetative growth. The level of ABA increases in vegetative tissues and regulates the expression of various stress-responsive genes under stress conditions. When plants are exposed to water deficiency, ABA accumulates in the roots and transfers to leaves through the transpiration stream. ABA concentration increases around guard cells and induces stomatal closure, which minimizes water loss and contributes to plant survival (Choi et al., 2013; Gao et al., 2011; Kang et al., 2002; Lee an Luan, 2012; Luan, 2002; Schroeder et al., 2001; Zeevaart and Creelman, 1988).

ABA-responsive gene expression is regulated by many transcription factors (TFs) that are induced by abiotic stresses and these TFs also mediate defense responses. For example, basic leucine zipper (bZIP) TFs interact with specific ABA-responsive elements (ABREs), which are cis-acting elements containing a (C/T)ACGTGGC consensus sequence that is present in the promoter region of ABA-regulated genes. Therefore, these TFs are termed ABRE-binding factors (ABFs) and the expression of their genes (*ABF1* to *ABF4*) is required for ABA production and stress responses (Choi et al., 2000; Finkelstein et al., 2005; Gao et al., 2011; Hossain et al., 2010; Jia et al, 2015; Kang et al., 2002; Wang et al., 2016). Constitutive expression of *AtABF3* was found to enhance drought tolerance in *Arabidopsis* (Abdeen et al., 2010; Kang et al., 2002; Kim et al., 2004), rice (Oh et al., 2005), tobacco (Kim et al., 2004), lettuce (Vanjildorj et al., 2005), and creeping bentgrass (Choi et al., 2013). However, genetic transformation using *AtABF3* gene for drought tolerance has not been applied to soybean so far. Soybean could be an important target for genetic transformation of *AtABF3* gene, thinking of its standing as a major crop and sensitivity to drought. There was no report on the performance of the *AtABF3* gene in this important crop. Soybean is a drought-sensitive plant, and an estimated 40% reduction in yield occurs due to drought conditions (Manavalan et al., 2009; Specht et al., 1999). Considering the damage to soybean production from drought stress, there is a growing interest to develop varieties that are drought tolerant. It could contribute to the production of stress tolerant crop for the commercial exploitation in agriculture.

Genetic engineering technology offers a possible route to elucidating and overcoming stress effects in plants. Development of genetically modified technology has made a tremendous achievement in solving problems that were difficult to solve with conventional breeding. Advances in genomics have developed commercial cultivars with the utilization of genetic transformation. (Nakashima and Yamaguchi-Shinozaki, 2013; Pathan et al., 2010). Many useful genes have been introduced into soybean using *Agrobacterium*-mediated transformation based on the cotyledonary-node method (Hinchee et al., 1988) and recently improved by use of half-seeds (Kim et al., 2012; 2013; Paz et al., 2006).

In the present study, we generated soybean transgenic plants overexpressing *AtABF3* by *Agrobacterium*-mediated transformation and investigated the performance of *AtABF3* gene on drought and salt tolerance in one of major field crop. Transgenic research with major crop will provide valuable information of a certain gene, whose function has primarily been addressed in the model plant species *Arabidopsis*, for its applicability to crop plants. Careful evaluation of transgenic soybean may lead to its commercialization or other practical uses in agriculture.

MATERIALS AND METHODS

Vector construction and *Agrobacterium* preparation

The *AtABF3* cDNA open reading frame was amplified from its original vector (provided by Dr. S Y Kim at Cheonnam National University, Korea) using an *AtABF3* forward primer (5′-CACCATGGGGTCTAGATTAAACTT-3′) and reverse primer (5′-CTACCAGGGACCCGTCAATG-3′). The resulting PCR product was subcloned into a pENTR/D-TOPO vector (Invitrogen, USA) and then transferred into a destination vector pB2GW7.0 (VIB-Ghent University, Ghent, Belgium) using LR clonase (Invitrogen, USA). The clone containing the recombinant plasmid was selected on an LB plate containing spectinomycin (50 mg l^{-1}). The plasmid, pB2GW7.0- *AtABF3* (Fig. 1A), was transformed into *Agrobacterium tumefaciens* strain EHA105 (Karimi et al., 2002) and cultured on solid YEP medium (10 g l^{-1} yeast extract, 5 g l^{-1} NaCl, 10 g l^{-1} peptic peptone and 1.0% plant agar, pH 7.0) containing 50 mg l^{-1} spectinomycin and 25 mg l^{-1} rifampicin at 28℃ for 2 days. A single colony was obtained and grown in 20 ml liquid YEP medium containing 50 mg l^{-1} spectinomycin and 25 mg l^{-1} rifampicin for 20 h at 28℃, at 250 rpm, until OD_{600} between 0.6 and 0.8 was attained. Competent cells were then prepared by adding an equal volume of 30% glycerol. Aliquots of competent cells were frozen and kept at -70℃.

Soybean transformation

Mature soybean seeds of the Korean cultivar 'Kwangankong' were used for transformation by following the method described by Kim et al. (2017). Plants with two trifoliate leaves were screened using a herbicide assay to identify transformants that expressed the *Bar* gene. The upper surface of a leaf was painted across the midrib with 100 mg l^{-1} PPT and Tween 20. The response to the herbicide was screened at 3-5 days after PPT painting. Plants undamaged by PPT were

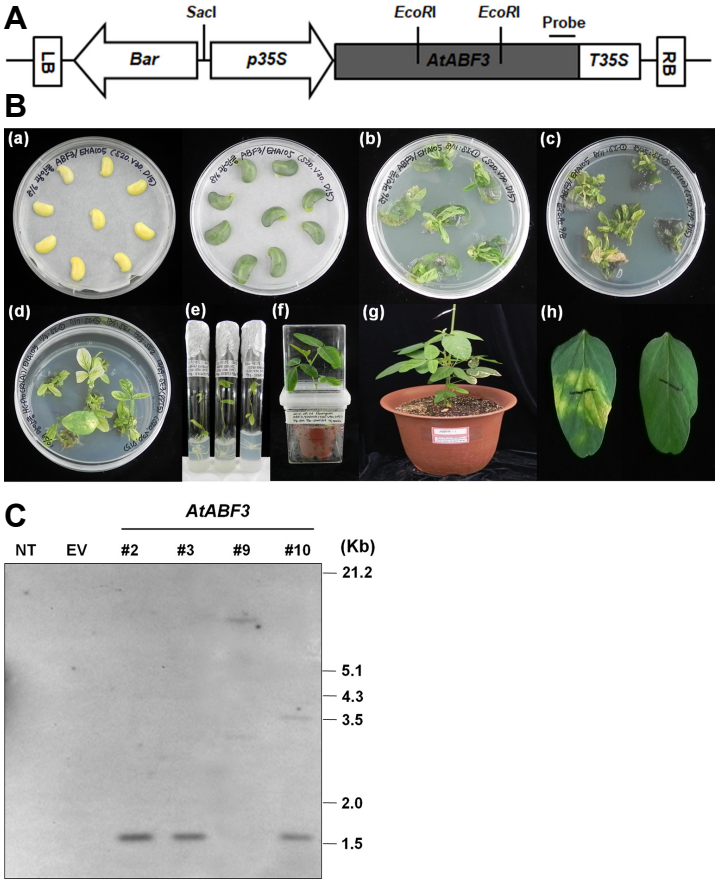

Fig. 1. Production of soybean transgenic plants with *AtABF3* gene using *Agrobacterium*-mediated transformation of half-seed explants. (A) Vector used for soybean transformation. Amplified *AtABF3* (1,365 bp size) was subcloned into pB2GW7.0 vector for soybean transformation. LB/RB, left/right T-DNA border; *p35S*/*T35S*, CaMV (cauliflower mosaic virus) 35S promoter/terminator; *Bar*, coding region of the DL-phosphinothricin resistance gene. Probe used for Southern blot analysis is also indicated. *Sac*I and *Eco*RI restriction enzyme sites are marked. (B) Production of *AtABF3* transgenic soybean plants. (a) Co-cultivation of half-seed explants after inoculation (left) and at 5 days after inoculation (right). (b) Shoot induction on SIM without PPT for 14 days. (c) Shoot induction on SIM including 10 mg l^{-1} PPT for another 14 days. (d) Shoot elongation on SEM including 5 mg l^{-1} PPT. (e) Root formation. (f) Acclimation of putative transgenic plant in a small pot. (g) Transgenic plant (T_0) grown in a large pot in the greenhouse. (h) Leaf painting with herbicide (100 mg l^{-1} PPT) showing sensitivity in NT plant (left) and resistance in transgenic plant (right). (C) Genomic Southern blot analysis of *AtABF3* transgenic soybean. Ten micrograms of genomic DNAs were digested with *Eco*RI and hybridized with probe *AtABF3*. The approximate DNA size markers are indicated on the *right*. NT, non-transgenic; EV, transformed with empty vector carrying only *Bar*; #2, #3, #9 and #10, *AtABF3* transgenic lines (T_2).

grown in greenhouse until maturity and seeds were harvested (Fig. 1B).

Confirmation of transgene in transgenic plants

Total genomic DNA was extracted from NT (non-transgenic) and transgenic plants using cetyltrimethyl ammonium bromide. A polymerase chain reaction (PCR) analysis was performed to detect *AtABF3* (5′-ATGGGGTCTAGATTAAACTT-3′/5′-CTACCAGGGACCCGTCAATG-3′) and *Bar* (5′-CATGTA ATGCTGCTCAAGGTACGC-3′/5′-ATGTAATGCTGCTCAAGG TACGC-3′); this PCR is expected to yield amplification products of 1,365 bp and 550 bp, respectively. To confirm the insertion of T-DNA, DNA from the left border to the *Bar* gene was amplified using the primer set 5′-TGGCTGGTGG CAGGATATATTGTG-3′/5′-AGACAAGCACGGTCAACTTCCG TA-3′ and DNA from the *AtABF3* gene to the right border was amplified using 5′-GGGACTAGTCTTCCATTGACTAGG-3′/5′-TTAAACTGAAGGCGGGAAACGACA-3′). Amplifications were performed using KOD FX (TOYOBO, Japan) according to the manufacturer's instructions with a thermal cycler (Bio-Rad, USA).

For Southern blot analysis, 10 μg of the genomic DNAs from NT and transgenic plants were digested overnight using *Eco*RI, for which a target site is absent in the *AtABF3* insert, fractionated on a 0.8% agarose gel by electrophoresis and transferred onto a Hybond N+ nylon membrane (Amersham Pharmacia, USA). Hybridization, washing, and detection were performed using a digoxigenin (DIG)-labeled DNA probe and a chemiluminescent system (Roche, Germany) according to the manufacturer's instructions. The DIG-labeled probe was prepared by PCR amplification of *AtABF3* using the primer set 5′-AGGGAATCAGCTGCAAGATC-3′/5′-AGCATTGCCTTTTGCATCCC-3′ that generates a fragment of 190 bp (Fig. 1A).

Analysis of RNA expression in transgenic plants

Total RNAs were isolated from NT and transgenic plants using the Plant RNA Purification Reagent (Invitrogen, USA) according to the manufacturer's instructions. Reverse transcriptase PCR (RT-PCR) was performed using the Maxime RT-PCR Premix (iNtRon, Korea) according to the manufacturer's instructions. The primers used in the RT-PCR for gene expression were as follows: *AtABF3* forward, 5′-GGGACTA GTCTTCCATTGACTAGG-3′/reverse, 5′-CAACAAACCCATTA CTAGCTGTCC-3′; *Bar* forward, 5′-CATGTAATGCTGCTCAA GGTACGC-3′/reverse, 5′-ATGTAATGCTGCTCAAGGTACGC-3′. The constitutively expressed gene *TUB* was used as a control and amplified using the primer set 5′-TGAGCAGT TCACGGCCATGCT-3′/5′-TCATCCTCGGCAGTGGCATCCT-3′).

Quantitative real-time PCR (qRT-PCR) was performed in 96-well plates with the CFX-96™ Real-Time system (Bio-Rad, Hercules, CA, USA). First-strand cDNA was synthesized using

Superscript™ II Reverse Transcriptase (Invitrogen) and oligo-dT (Invitrogen) following the users' instruction manual. The quantity and quality of the synthesized cDNAs were determined by spectrophotometry. Each reaction contained 3 μl (3 ng/μl) of cDNA, 0.5 μl (10 pm/μl) of each primer and 10 μl SYBR® Premix Ex Taq™ (Takara) in a total reaction volume of 20 μl. The PCR conditions were as follows: 95℃ for 3 min; 40 cycles of 10 s at 95℃, 10 s at 55℃, and 20 s at 72℃. A dissociation curve was generated by increasing the temperature from 65 to 95℃ to check for amplification specificity. The efficiency and standard deviation of each primer were obtained using Bio-Rad CFX Manager v1.6.541.1028 on a standard curve generated from a two-fold dilution series of one sample at five dilution points for two technical replicates. Baseline and threshold cycle (Ct value) were automatically calculated with default parameters. Expression of *AtABF3* was analyzed using the following primers: forward, 5′-AGGGAATCAGCTGCAAGATC-3′ and reverse, 5′-AGCATTG CCTTTTGCATCCC-3′. *TUB* was used as the internal control to normalize the amount of leaf RNA in the soybeans (Hu et al., 2009).

Stress treatment of transgenic plants

For drought tolerance analysis, NT, EV (transformed with empty vector carrying only *Bar*), and transgenic plants were grown in the same volume of soil and identical containers in a growth chamber at 25℃, with a long day photoperiod (18 h light/ 6 h dark), and 60% humidity for 3 weeks until the leaves on 2 nodes were fully expanded. Under the same conditions, water was withheld from plants for 20 days before watering was recommenced.

For salt tolerance analysis, NT, EV, and transgenic plants were grown on wet rock wools (Urmedia, Korea) in the growth chamber. NT, EV, and transgenic plants with fully expanded leaves on 2 nodes were transplanted into Hoagland's solution containing 200 mM NaCl for 11 days to induce salt stress.

Determination of relative ion leakage and total chlorophyll

The extent of ion leakage from NT and transgenic plants after stress treatment was investigated by measuring conductivity. Leaf samples (1 g) were soaked in 10 ml of distilled water for 24 h at room temperature and the conductivity of the solution (L_t) was measured using a EC-400L conductivity meter (Istek, Korea). The leaf samples were then returned to the solution in the tubes, which were sealed and incubated at 95℃ for 20 min. The solution (L_0) was then cooled to room temperature and conductivity was re-measured. The $L_t/L_0 \times 100$ values were calculated and used to evaluate relative electrolyte leakage (Fan et al. 1997). Statistical analysis was also performed using the Excel *t*-test program to confirm significant differences. Asterisks indicate significant changes compared to NT plants ($^{*}P < 0.05$; $^{**}P < 0.01$).

Total chlorophyll from leaves of NT and transgenic plants after stress treatment was isolated in 80% acetone (v/v). The chlorophyll content was calculated spectrophotometrically as described by Wu (2008). Statistical analysis was also performed using the Excel *t*-test program to confirm significant differences. Asterisks indicate significant changes compared to NT plants ($^{*}P < 0.05$; $^{**}P < 0.01$).

Measurement of transpiration rate

NT, EV, and transgenic plants were grown in the same volume of soil and in identical containers in the growth chamber at 25℃, with a long day photoperiod (18 h light/ 6 h dark), and 60% humidity. Leaves at similar developmental stages (fully expanded leaves from 2 nodes) were detached and weighed on a sterile bench in an extractor hood. Leaf weight was measured at 40-min intervals for 200 min and compared to the initial weight. Statistical analysis was performed using the Excel *t*-test program to confirm significant differences. Asterisks indicate significant changes compared to NT plants ($^{*}P < 0.05$; $^{**}P < 0.01$).

Examination of stomatal closure

The behavior of stomatal guard cells was analyzed during water deficit conditions. NT, EV, and transgenic plants were grown in the same volume of soil and in identical containers in the growth chamber at 25℃, with a long day photoperiod (18 h light/6 h dark), and 60% humidity until leaves on 2 nodes were fully expanded. The plants were then subjected to water stress for 20 days. Leaves of similar developmental stage were detached from randomly chosen sites, fixed in 80% acetone, immersed in 5% NaOH, and boiled for 1 min. They were then washed three times with distilled water, and incubated in bleach until they had lost their color (Fu et al., 2002). Photographs were taken with the abaxial side up of leaves from plants after 7, 11, 15, and 20 days of water stress. The number of stomatal guard cells was counted and the stomata were assessed: completely open, partially open, and completely closed (Huang et al., 2009; Choi et al., 2013).

Investigation of agronomic traits in the greenhouse

NT and transgenic soybean seeds (T_2) were planted in a seedling tray and seedlings were kept in a greenhouse. Plants were watered either regularly (every 4 days) or rarely (every 10 days) to evaluate agronomic traits such as plant height, number of branches per plant, and number of nodes per plant. In addition, the number of pods per plant and the seed weight per plant were counted to investigate the relative yield of transgenic plants. Statistical analysis was performed using the Excel *t*-test program to identify significant differences. Asterisks indicate significant changes compared to NT plants ($^{*}P < 0.05$; $^{**}P < 0.01$).

RESULTS AND DISCUSSION

Production of *AtABF3* transgenic soybean plants

Recently, stable *Agrobacterium*-mediated soybean transformation (Kim et al., 2012; 2013) was established in our laboratory based on the half-seed (Paz et al., 2006) and cotyledonary-node methods (Hinchee et al., 1988). The *Agrobacterium*-mediated transformation method is a simple effective protocol for the production of transgenic soybean with a reliable insertion of a single or low copy numbers (Hansen and Wright, 1999; Li et al., 2017). Our protocol included a few modifications such as additional treatment

with thiol compounds (a mixture of L-cysteine, sodium thiosulfate and dithiothreitol). These antioxidants had the beneficial effect of reducing severe tissue browning or necrosis, and also enabled to promote the shoot growth from shoot pad, thus enhanced the transformation efficiency (Dan, 2008; Olhoft et al., 2003).

Compared with our previous study (Lee et al., 2006), the modified protocol significantly improved production of successful transgenic plants. The modified transformation protocol enabled generation of many different transgenic soybeans; to date, more than 20 different transgenic plants have been produced (data not shown).

To confirm the integration of the transgene in transformed soybean plants, genomic DNA was isolated from T_0 transformants and analyzed using PCR for the presence of *AtABF3* and *Bar*. Additionally, T-DNA insertion was verified by amplifying fragments between the left border and the *Bar* gene and right border and the *AtABF3* gene (Supplementary Fig. S1). Nine transgenic lines showed the expected amplification of transgene sequences.

The genetic stability of integrated genomic DNA was analyzed by Southern blotting using leaf samples from four T_2 seedlings (lines #2, #3, #9, and #10) to determine the copy number of transgenes (Fig. 1C). These four lines were selected because of their phenotype and the production of sufficient seeds. Genomic DNAs from NT, EV, and transgenic plants were digested with *EcoR*I and hybridized with an *AtABF3* probe. Transgenic lines #2 and #3 appeared to contain a single insertion (low copy number of the transgene), while lines #9 and #10 contained multiple insertions.

The expression of the transgenes was analyzed by reverse transcriptase-PCR (RT-PCR) using *Bar* and *AtABF3* primers (Supplementary Fig. S2). All the transgenic lines expressed the *Bar* gene; no expression was observed in NT plants.

Drought tolerance of *AtABF3* transgenic soybean plants

Overexpression of *AtABF3* has been demonstrated to enhance drought tolerance in *Arabidopsis*, rice, tobacco, lettuce, and creeping bentgrass (Choi et al., 2013; Kang et al., 2002; Kim et al., 2004; Oh et al., 2005; Vanjildorj et al.,

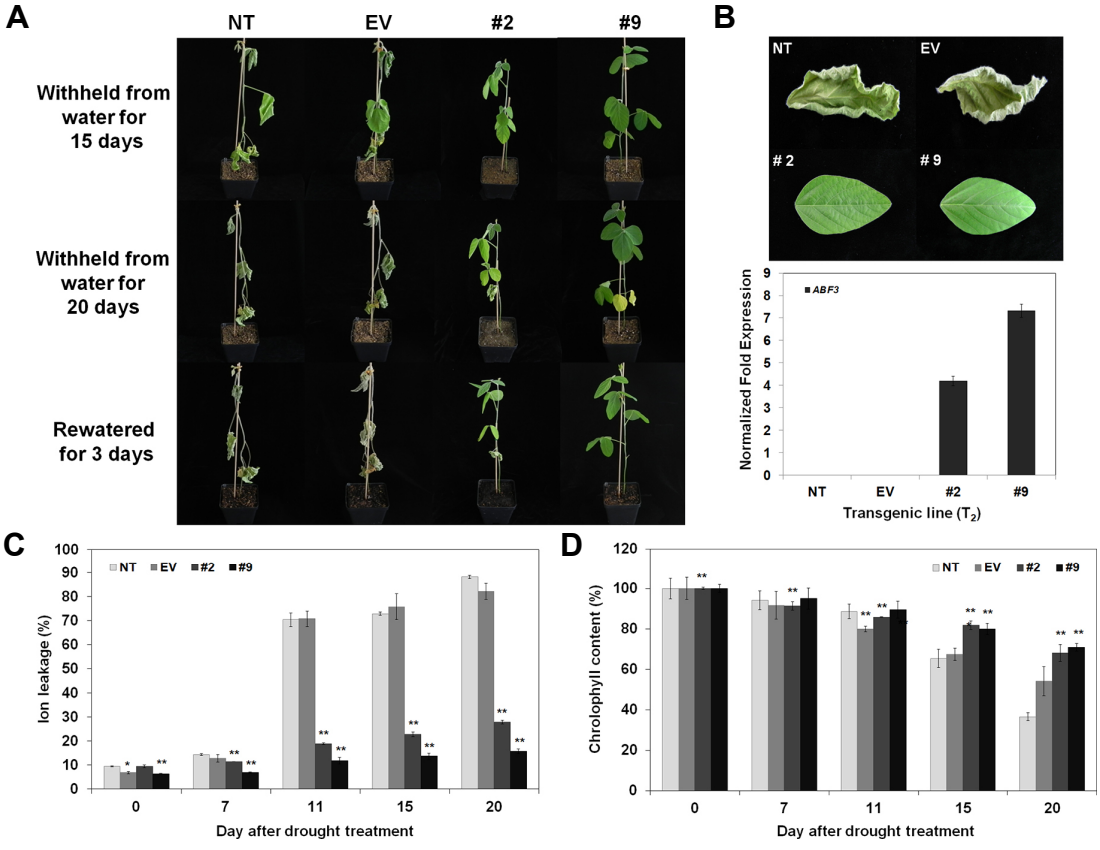

Fig. 2. Drought tolerance of *AtABF3* transgenic plants (T_2). (A) Drought tolerance analysis of *AtABF3* transgenic plants compared with NT and EV plants. Plants were grown on soil until leaves were fully expanded on 2 nodes, withheld from water for 20 days, and then re-watered (n = 12 each). The photographs were taken 15 and 20 days after drought treatment and 3 days after re-watering. (B) *AtABF3* gene expression (bottom) with detached leaves of 20 days after drought treatment (top) using quantitative real-time PCR (qRT-PCR). (C, D) Ion leakage and chlorophyll content were measured at the indicated days after drought treatment from 2-node leaves of NT, EV and transgenic plants (n = 6 each). NT, non-transgenic; EV, transformed with empty vector carrying only *Bar*; #2 and #9, transgenic lines (T_2). Error bars indicate mean ± standard deviation. Asterisks indicate significant changes compared with NT ($^*P < 0.05$; $^{**}P < 0.01$).

2006). In this study, we examined the drought stress tolerance of transgenic lines #2 and #9 and compared them with NT and EV plants. At the end of the 20-day drought treatment, the NT and EV plants had withered completely, whereas plants of lines #2 and #9 were less affected and survived the water deficit conditions. After 3 days of re-watering, plants of lines #2 and #9 had fully recovered and started to grow; in contrast, NT and EV plants did not respond to re-watering and eventually died (Fig. 2A). The leaves of line #2 plants appeared more withered than those of line #9 plants, but leaves of both transgenic lines showed much less withering than those of NT and EV plants. We concluded that line #9 plants showed the greatest drought tolerance of the plant lines analyzed. The relationship between *AtABF3* gene expression levels and drought-tolerance was investigated by qRT-PCR (Fig. 2B). *AtABF3* gene expression was confirmed in the two transgenic lines (#2 and #9); as expected, no expression was detected in the NT and EV plants. The relative level of expression was higher in line #9 (high transgene copy number) than line #2 (low transgene copy number) (Fig. 2B). Our results indicate that the level of *AtABF3* expression was correlated with the phenotypic differences among the transgenic and non-transgenic lines. Previous studies have similarly concluded that higher expression of *AtABF3* in transgenic plants results in increased drought tolerance (Choi et al., 2013; Kang et al., 2002).

The physiological aspects of enhanced drought tolerance in the transgenic lines were analyzed by measuring changes in ion leakage and chlorophyll content. Drought stress resulted in a significant increase in ion leakage from leaves of NT and EV after 11 days of treatment. Transgenic lines #2 and #9 showed increased ion leakage but at a significantly lower level ($P < 0.01$) than NT and EV plants (Fig. 2C). In addition, the chlorophyll contents of the leaves of transgenic lines #2 and #9 were significantly higher than those of NT and EV plants ($P < 0.01$) (Fig. 2D). These results suggest that drought-tolerant lines (#2 and #9) were less affected by water deficit conditions and that their enhanced drought tolerance was due to the prevention of cell membrane damage and maintenance of chlorophyll content.

Water loss of transgenic plants by transpiration was compared to that from NT and EV plants by weighing leaves immediately after detachment and at intervals over the following 200 min (Fig. 3A). At 200 min after detachment, the water content of NT and EV plant leaves fell to about 55% and 48%, respectively, of the start level. In contrast, those of transgenic lines #2 and #9 decreased to 69% and 72%, respectively (Fig. 3B). Thus, water loss from transgenic lines #2 and #9 was slower than from NT and EV plants ($P < 0.01$). The increased drought-tolerance of lines #2 and #9 appeared to be associated with a lower transpiration rate.

The ratios of stomatal closure in NT, EV, and transgenic lines #2 and #9 were compared during the drought treatment (Choi et al., 2013; Kang et al., 2002). Stomatal guard cell behavior was analyzed microscopically using detached leaves (data not shown). The degree of stomatal opening was classified as completely open, partially open, or completely closed (Choi et al., 2013; Huang et al., 2009). At the end of the 20-day drought treatment, 3.5% and 56% of the stomata of transgenic lines #2 and #9 were completely closed, respectively. Approximately 44% of stomata in line #2 were completely open, whereas only 16% were completely open in line #9. Almost all stomata were completely open in NT and EV plants. In line #2, 52% of stomata were

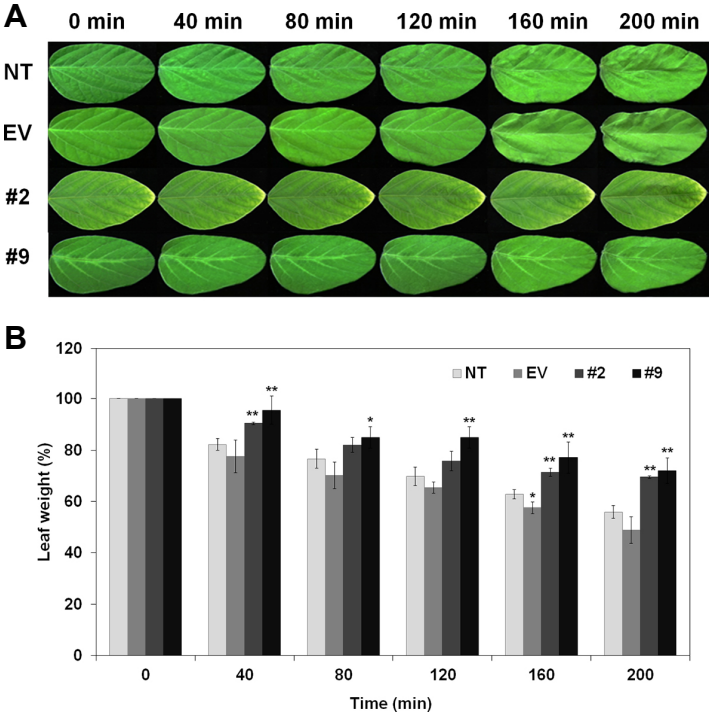

Fig. 3. Transpiration rate of *AtABF3* transgenic plants (T_2). Plants were grown on soil until leaves were fully expanded on 2 nodes, leaves were detached (n = 6) and weighed at the indicated times (A, B). The photographs were taken at the indicated times after drought treatment. NT, non-transgenic; EV, transformed with empty vector carrying only *Bar*; #2 and #9, transgenic lines (T_2). Error bars indicate mean ± standard deviation. Asterisks indicate significant changes compared with NT ($^{*}P < 0.05$; $^{**}P < 0.01$).

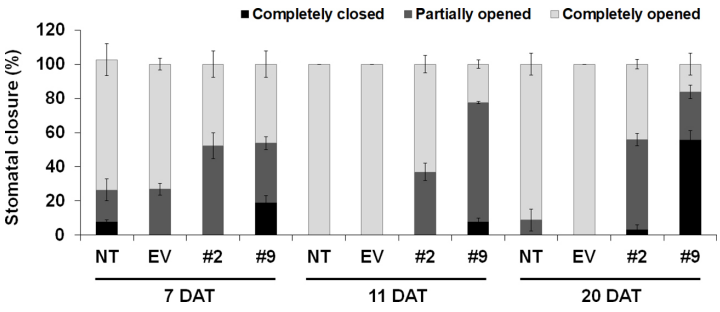

Fig. 4. Stomatal aperture of *AtABF3* transgenic plants (T_2). Stomatal guard cells were observed using a microscope at the indicated days after drought treatment (DAT) from 2-node leaves of NT, EV and *AtABF3* transgenic plants (n = 6 each). The ratios of stomatal closure of NT, EV and *AtABF3* transgenic plants were measured. The stomata were assessed as completely open, partially open, and completely closed (n = 90 each). NT, non-transgenic; EV, transformed with empty vector carrying only *Bar*; #2 and #9, transgenic lines (T_2). Arrows indicate guard cells. Error bars indicate mean ± standard deviation.

partially open, whereas only 27% were partially open in line #9 (Fig. 4). Our observations indicate that drought tolerance in *AtABF3* transgenic lines #2 and #9 was associated with higher rates of stomatal closure, which reduced plant water loss.

Guard cells are regarded as a good model system for understanding signal transduction in plants. Under a water deficit, plants synthesize ABA, which triggers stomatal closure, even in daytime. Our data confirmed that *AtABF3* was involved in an ABA-controlled process, namely drought-induced stomatal closure (Luan, 2002; Schroeder et al., 2001; Wang et al., 2011). Stomatal closure following *AtABF3* gene expression was reported in *Arabidopsis* (Kang et al., 2002) and creeping bentgrass (Choi et al., 2013). On the basis of these results, we suggest that stomatal closure in soybean leads to improved drought tolerance.

Salt tolerance of *AtABF3* transgenic soybean plants

Several studies have shown that overexpression of bZIP transcription factor genes enhances the response of transgenic plants to ABA and triggers stomatal closure under stress conditions, resulting in improved tolerance of drought and high salt conditions (Gao et al., 2011; Hossain et al., 2010; Jia et al., 2015; Wang et al., 2016; Yang et al., 2011).

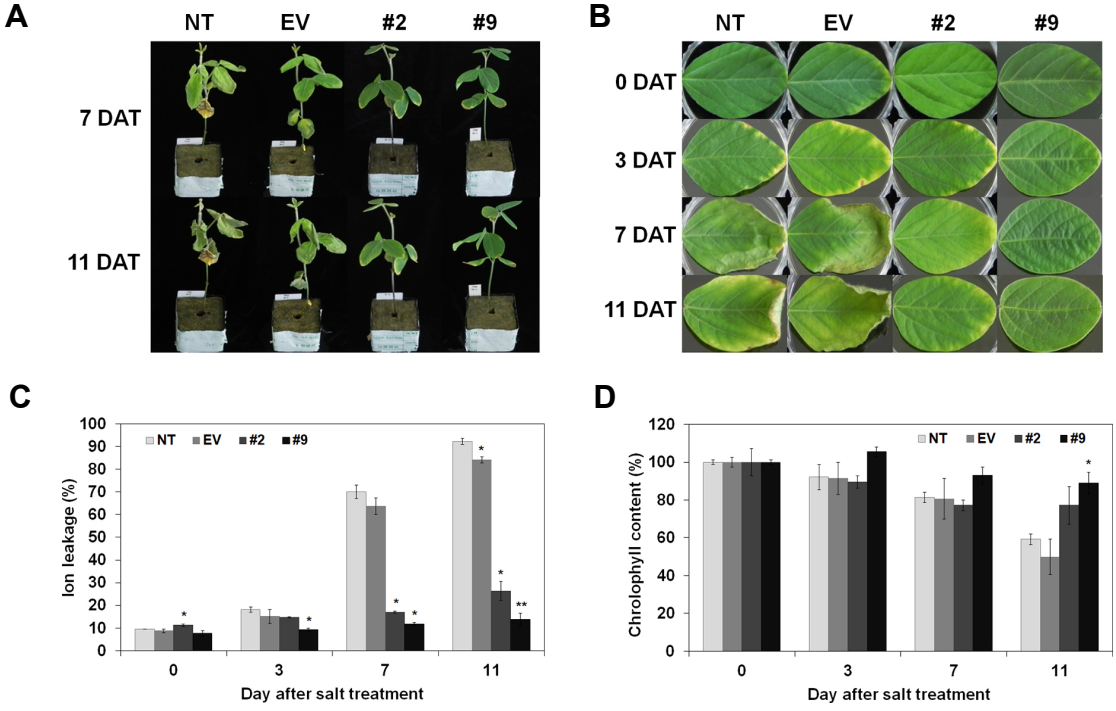

Fig. 5. Salt tolerance of *AtABF3* transgenic plants (T_2). (A, B) Salt tolerance analysis of *AtABF3* transgenic plants compared with NT and EV. Plants were grown on wetted rock wool until leaves were fully expanded on 2 nodes, and then soaked in 200 mM NaCl solution for 11 days (n = 12 each). The photographs were taken 7 and 11 days after salt treatment. (C, D) Ion leakage and chlorophyll content were measured at the indicated days after salt treatment from leaves of NT, EV and transgenic plants (n = 6 each). NT, non-transgenic; EV, transformed with empty vector carrying only *Bar*; #2 and #9, transgenic lines (T_2). Error bars indicate mean ± standard deviation. Asterisks indicate significant changes compared with NT ($^*P < 0.05$; $^{**}P < 0.01$).

Here, we compared the salt tolerance of NT, EV, and line #2 and #9 plants grown in 200 mM NaCl solution for 11 days and also examined whether expression of *AtABF3* was correlated with salt tolerance (Fig. 5). After 7 days of salt treatment, the leaves of NT and EV plants began to wilt and changed color to yellow at their edges; leaves of lines #2 and #9 were much less affected by the treatment and remained green (Figs. 5A and 5B). The apparently enhanced salt tolerance of transgenic lines was investigated by measuring ion leakage rates and chlorophyll contents. Salt stress resulted in an increased rate of ion leakage from NT and EV leaves after 7 days of salt treatment. In contrast, ion leakage rates only increased slightly in lines #2 and #9 at this time and were significantly lower than those of the NT plants ($P < 0.05$ in #2 and $P < 0.01$ in #9) (Fig. 5C). Chlorophyll contents of leaves in lines #2 and #9 were higher than those of NT and EV plants after 20 days of stress treatment (Fig. 5D). On the basis of these results, we suggest that transgenic lines #2 and #9 were not significantly affected by salt treatment due to the prevention of cell membrane damage and maintenance of chlorophyll content.

Salt stress, similar to other important abiotic stresses such as drought, cold, and flooding, also inhibits plant growth and crop yield. When plants are exposed to salt stress, extraction of water from the soil to their roots is hampered by the high osmotic pressure in the soil solution. Moreover, plants are also damaged by the accumulation of sodium and chloride (Zhu, 2001; Pathan et al., 2010). This analysis confirmed that drought and salt tolerance was conferred by expression of *AtABF3* in transgenic soybeans.

Investigation of agronomic traits of *AtABF3* transgenic plants in the greenhouse

To examine the agronomic characteristics of *AtABF3* transgenic plants (T_2), we compared their growth characteristics to those of NT plants under conditions of regular watering (every 4 days) and low rate of watering (every 10 days) (Fig. 6). Agronomic traits including plant height, the number of nodes per plant, branches per plant, pods per plant and total seed weight of NT plants and drought-tolerant line (#9) were investigated in greenhouse. The number of seeds of line #2 was insufficient, so that line #9 plants were only analyzed for the agronomic traits. Under regular watering conditions, line #9 plants were smaller than NT plants, had fewer pods, and produced a lower total seed weight (Fig. 6A). Under the low rate of watering, line #9 plants were smaller than NT and the number of pods was also lower. However, total seed weight in line #9 was significantly higher than in NT plants ($P < 0.01$) (Fig. 6B). Under low watering rate conditions, the pods of NT plants showed severe withering, and most of the pods failed to set normal seeds. Transgenic plants of line #9 showed better seed filling under the low watering rate conditions. During reproductive growth, water stress can cause a reduction of yield due to retardation of growth. Soybean plants that were continuously exposed to water stress show greater nitrogen and chlorophyll loss from leaves than non-stressed plants. This shortens the seed-filling period, so that smaller seeds and reduced yield occurs under drought conditions (Manavalan et al., 2009).

In this study, we introduced the *AtABF3* gene from *Arabidopsis* into soybean via *Agrobacterium*-mediated trans-

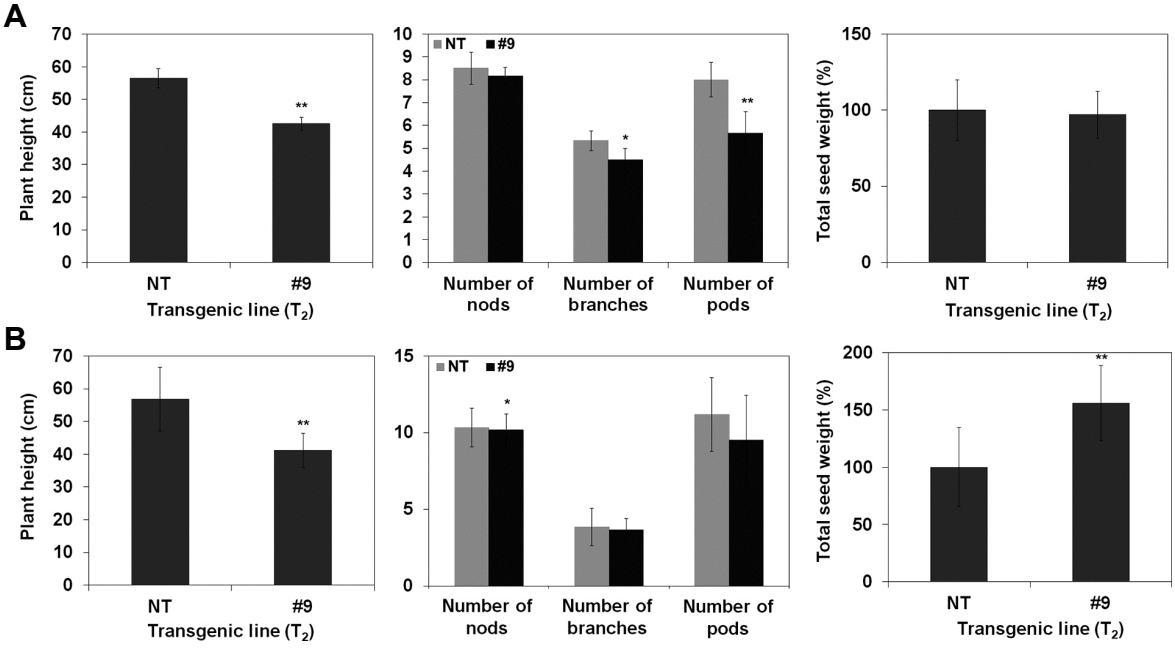

Fig. 6. Agronomic characters of non-transgenic (NT) and transgenic plants (T_2) grown in the greenhouse. NT and T_2 plants were grown in the greenhouse, and agronomic characters were investigated in regularly watered conditions ($n = 8$ each) (A) and in rarely watered conditions ($n = 8$ each) (B). NT, non-transgenic; #9, transgenic (T_2) plant. Error bars indicate mean ± standard deviation. Asterisks indicate significant changes compared with NT ($^*P < 0.05$; $^{**}P < 0.01$).

formation. Overexpression of the ectopic gene conferred drought and salt tolerance compared to non-transgenic plants. One of concern is the involvement of *AtABF3* in stomatal closure. Complete or partial closure of stomata enhances drought tolerance by inhibition of water loss. However, closure of stomata can have negative effects on air supply to the leaf. Possibly, the smaller plant height of transgenic plants was associated with the effects on air supply due to stomatal closure. For better application of this gene, the timing of its expression needs to be controlled using more sophisticated molecular designs, such as inducible promoter-driven gene expression. The significance of our work is to test a gene with known function from model plant in field crop soybean. Even though we couldn't deploy transgenic soybean to wider field level, transgenic research via soybean transformation in major crop is necessary to explore the practical potential of newly-identified gene.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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