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ORIGINAL PAPER

Overexpression of an *Arabidopsis* β-glucosidase gene enhances drought resistance with dwarf phenotype in creeping bentgrass

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Abstract An *Arabidopsis* β-glucosidase, AtBG1 is known to hydrolyze glucose-conjugated, biologically inactive abscisic acid (ABA) to produce active ABA, which increases the level of ABA in plants. Since an increase of ABA in plants confers tolerance against abiotic stress such as drought, we introduced the pCAMBIA3301 vector harboring the *AtBG1* gene into creeping bentgrass through *Agrobacterium*-mediated transformation. After transformation, putative transgenic plants were selected using the BASTA resistance assay at a concentration of 0.8 %. Genomic integration of the *AtBG1* gene was confirmed by genomic PCR and Southern blot analysis, and gene expression was validated by Northern blot and Western blot analyses. Interestingly, the transgenic bentgrass plants overexpressing *AtBG1* had a dwarf phenotype with reduced growth rates when compared to wild-type creeping bentgrass. In addition, the transgenic plants accumulated higher ABA levels and displayed enhanced drought tolerance. These results suggest that the expression of AtBG1 in plants induces the accumulation of higher ABA levels, which results in the formation of dwarf creeping bentgrass and enhances the survival in water-limiting environments. *Key message* We used an *Arabidopsis* β-glucosidase AtBG1 to engineer a crop with elevated active ABA levels, and developed transgenic creeping bentgrass with enhanced drought tolerance and dwarf phenotype.

Keywords AtBG1 · β-Glucosidase · Creeping bentgrass · Transformation · Drought tolerance · Dwarf

Introduction

Creeping bentgrass (*Agrostis stolonifera* L.) is the principal cool-season turfgrass species used for putting greens in golf courses, since it is characterized by fine texture, dense growth, and tolerance to low cutting heights (Warnke 2003). Creeping bentgrass often requires daily irrigation and frequent mowing because of its intolerance to drought stress and vigorous growth, both of which add to maintenance costs in the management of golf courses. Therefore, methods that increase drought resistance and reduce the frequency of mowing while maintaining turfgrass quality are desirable. In this regard, plant transformation represents a rapid and effective approach to produce turfgrass varieties with drought resistance and reduced mowing when compared with traditional breeding (Zilinskas and Wang 2004). The first genetic transformation of creeping bentgrass was achieved by microprojectile bombardment of the embryogenic callus (Zhong et al. 1993), while *Agrobacterium*-based protocols have been established more recently (Luo et al. 2004; Yu et al. 2000). We also developed *Agrobacterium*-mediated transformation methods of creeping bentgrass, which were used to develop herbicide-resistant, purple-colored or disease-

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resistant bentgrass varieties (Cho et al. 2011; Han et al. 2009; Kim et al. 2007).

The phytohormone abscisic acid (ABA) is critical for growth, development, and survival of plants in response to environmental stresses. Plants have to adjust ABA levels constantly to respond to changing physiological and environmental conditions (Nambara and Marion-Poll 2005). The level of this hormone is regulated by the relative rates of biosynthesis, catabolism, conjugation, and redistribution throughout the plant. In particular, ABA levels increase when plants are subjected to abiotic stress and decrease when rescued from stressed conditions. Mutant plants deficient in ABA biosynthesis are typically unable to control their stomatal behavior appropriately in response to water stress, leading to a wilty phenotype, whereas genetically engineered plants with increased ABA levels display enhanced stress tolerance (Xiong and Zhu 2003). Thus, engineering plants with elevated ABA levels and subsequent stress tolerance has been achieved by manipulating the key enzymes in ABA biosynthesis, including zeaxanthin epoxidase (ZEP) and 9-*cis*-epoxycarotenoid dioxygenase (NCED). Overexpression of *NCED* in transgenic plants has shown to result in the high accumulation of endogenous ABA, which enhances ABA responses with stress tolerance (Aswath et al. 2005; Iuchi et al. 2001; Qin and Zeevaart 2002; Thompson et al. 2000, 2007; Tung et al. 2008). It has been also reported that overexpression of *ZEP* enhances tolerance to osmotic stress due to increased ABA levels (Park et al. 2008). Therefore, ABA levels in plants can be manipulated by overexpressing key regulatory genes in ABA biosynthesis and thus stress tolerance can be improved by increasing ABA levels.

In the regulation of ABA levels in plants, ABA is also conjugated with glucose, resulting in the formation of an ABA glucose ester (ABA-GE) which is one of the major inactive forms of ABA and is widespread in the plant kingdom (Sauter et al. 2002). Although the formation of ABA-GE was previously considered an inactivation mechanism for ABA, an *Arabidopsis* β-glucosidase, AtBG1 (At1G52400) was shown to hydrolyze glucose-conjugated, biologically inactive ABA to produce active ABA, which contributes to increased cellular ABA levels under dehydration conditions (Lee et al. 2006). This report demonstrated that the cleavage of ABA-GE by the ABA-specific β-glucosidase (i.e., AtBG1) could be used as a way to produce bioactive ABA in response to dehydration stress and be exploited as a new route for quick release of ABA in response to changes in stress conditions (Schroeder and Nambara 2006). Thus, the *atbg1* mutant displayed ABA-deficient phenotypes, whereas transgenic *Arabidopsis* plants harboring *AtBG1* under the control of the 35S promoter exhibited enhanced tolerance to drought and salt stresses (Lee et al. 2006). Therefore, overexpression of AtBG1 in plants might confer stress tolerance by increasing the biologically active ABA levels from inactive ABA-GE.

In the present study, we introduced the *AtBG1* gene into creeping bentgrass via *Agrobacterium*-mediated transformation to investigate the phenotypic effects of AtBG1 overexpression and to develop a stress-resistant bentgrass variety. Interestingly, the transgenic plants overexpressing AtBG1 displayed dwarf phenotypes with greatly reduced growth, as well as drought stress tolerance. Therefore, these results demonstrated that it is possible to manipulate bioactive ABA levels in plants by overexpressing the ABA-GE cleavage enzyme and that drought tolerance can be improved in creeping bentgrass by increasing the ABA levels via AtBG1 overexpression.

Materials and methods

Gene construct

From the hemagglutinin (HA)-tagged *AtBG1* (*AtBG1:HA*) construct used in the previous report (Lee et al. 2006), the *AtBG1:HA* gene was subcloned into the binary vector pCAMBIA3301 with *Bam*HI and *Sma*I. The maize ubiquitin promoter and *Arbcs* transcription terminator were used to drive the expression of *AtBG1*. The pCAMBIA3301 construct carries an intron-containing β-glucuronidase (*intron-gus*) gene as a reporter along with the *bar* gene for herbicide resistance as a selectable marker and both of these genes are under the control of the cauliflower mosaic virus (CaMV) 35S promoter. The integrity of the construct was confirmed by DNA sequencing. The plasmid construct was transformed into the *Agrobacterium tumefaciens* strain EHA105 using the freeze–thaw method (Chen et al. 1994), followed by bentgrass transformation.

Generation of putative transgenic bentgrass

Seeds for the "Crenshaw" cultivar of creeping bentgrass (*A. stolonifera* L.) were purchased from KVBio Inc., Korea, and stored at 4 °C before use. Tissue culture and genetic transformation of creeping bentgrass were performed as previously described (Han et al. 2009; Kim et al. 2007). During the transformation process, GUS staining assays were performed to check whether or not the embryogenic calli were transformed. After transformation, plantlets with well-developed roots were transferred to soil, grown under greenhouse conditions for 2 weeks, and then sprayed with 0.8 % (v/v) BASTA® (which contains 18 % glufosinate ammonium) to select for transgenic bentgrass plants. Herbicide resistance was determined 10 days later, and herbicide-resistant plants were further analyzed.

Molecular analysis of transgenic bentgrass plants

Southern blot and Northern blot analyses of transgenic bentgrass plants were performed as previously described (Han et al. 2009; Kim et al. 2007). Total genomic DNA was isolated from the leaves of greenhouse-grown plants. For the Southern blot analysis, the *bar* or *AtBG1* gene probe was labeled with [α^{32}P] dCTP using the Radiprime™ II Random Prime Labeling System (Amersham Biosciences, UK). Genomic DNA was digested with either *Hin*dIII or *Eco*RI for the Southern blot analysis with *bar* probe, and with *Bam*HI or *Xba*I for the Southern blot analysis with *AtBG1* probe. For Northern blot analysis, total RNA was extracted from the leaves using Trizol® reagent (Invitrogen, CA), and hybridizations were carried out with [α^{32}P] dCTP-labeled *bar* or *AtBG1* probes.

For Western blot analysis, several leaves were taken, ground, and extracted with extraction buffer (70 mM Tris–HCl, pH 8.3, 7 mM EDTA, 35 % ethylene glycol, 98 mM ammonium sulfate, 14 mM sodium metabisulfite, 0.07 % polyethyleneimine and 2.8 mM PMSF). The extracted protein samples were centrifuged at 14,000 rpm for 15 min at 4 °C, and 30 μg of each protein sample was loaded onto 10 % SDS-PAGE gels for electrophoresis. The protein bands on the SDS-PAGE gel were transferred to a polyvinylidene difluoride (PVDF) membrane (Hybond-P; Amersham Biosciences, UK), and the membrane was then incubated with HA-specific antibody (Santa Cruz Biotechnology, CA) to detect HA-tagged AtBG1 for 2 h and developed using an ECL™ Western blotting analysis system (Amersham Biosciences, UK).

Phenotypic analysis of transgenic bentgrass plants

Creeping bentgrass plants in soil were grown and vegetatively propagated routinely in a culture room (22–24 °C with a 16 h photoperiod). For the phenotypic analyses, the transgenic bentgrass plants were grown to similar stages and trimmed to the same heights, and then the trimmed plants were further grown for 4 weeks in the culture room. Non-transgenic wild-type bentgrass (NT) and transgenic plant with pCAMBIA3301 (i.e., herbicide-resistant bentgrass; HR) were also included as controls. Leaf lengths were measured from the second leaf of the first stoloniferous plant, and leaf widths were measured on the widest part from the second leaf ($n = 5$). The relative leaf widths were then calculated by setting the leaf width of non-transgenic bentgrass to 1. To investigate the growth rates of transgenic bentgrass plants, leaf growth rates were evaluated by measuring leaf lengths every 4 days during 4 weeks of growth. Five leaves of each plant were used for the measurements, and the leaf growth rates (cm/4 days) were calculated from ((the leaf length of current measurement) − (the leaf length of previous measurement)) at the intervals of 4 days. The entire test was repeated at least three times and the results were consistent.

Quantification of ABA levels

To determine endogenous active ABA accumulation in transgenic bentgrass plants, 0.1 g of fresh weight of 4-week-grown leaves with or without dehydration treatment was used. The dehydration treatment was carried out by air-drying the detached leaves at room temperature for 2 h (i.e., condition with approximately 70 % of fresh weight and 30 % of dehydration), as described previously (Ren et al. 2007). The dehydrated samples were further incubated in the dark for 4 h at room temperature. The leaf samples with or without dehydration treatment were ground using a TissueRuptor® (Qiagen, GmbH) in liquid nitrogen and treated with 1 ml of ABA extraction buffer (80 % methanol (v/v), 2 % glacial acetic acid). The extract was then incubated for 24 h at 4 °C in the dark. After centrifugation, the supernatants were dried using an evaporator, and the pellets were resuspended in 100 μL of TBS with 10 % methanol. ABA concentrations of each sample were measured with the Phytodetect ABA test kit (Agdia Incorperated, USA) using the competitive antibody binding method, following the manufacturer's protocol. Experiments for each plant were performed three times and each sample was analyzed in duplicate for the ELISA.

Drought tolerance assay

In the drought tolerance assay, the bentgrass plants were grown to similar stages in 25 cm diameter pots and subjected to drought stress. We planted both non-transgenic control and transgenic plants in the same pots and examined their tolerance to drought in the same culture conditions. All pots were fully irrigated before the start of the drought test, and then drought-stressed by terminating irrigation. The leaves ($n \geq 5$) of the drought-stressed plants were harvested every 4 days and immediately weighed (FW, fresh weight), and then dried in an oven at 65 °C for 3 days and weighed (DW, dry weight). Drought tolerance was investigated by evaluating the water content (WC) of the tested plants, as previously reported (Bajji et al. 2000). The WC was the amount of water in the leaf relative to its DW. The water content was measured using the following formula: $WC = ((FW - DW)/(FW)) \times 100\ \%$. After 4 weeks of drought treatment, the plants were re-irrigated and the relative WC was measured after re-irrigation to examine plant recovery. The drought tolerance assays were repeated at least three times with consistent results.

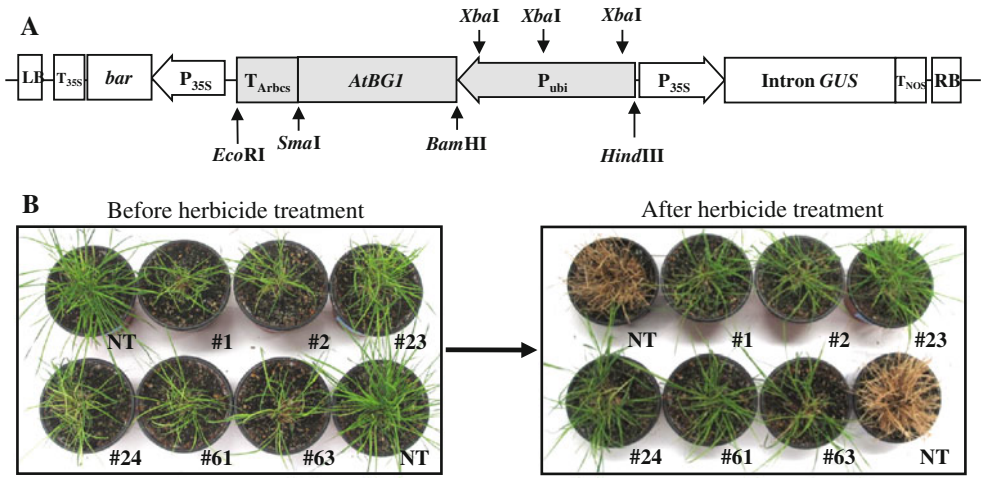

Fig. 1 Construct for the transformation and herbicide resistance assay of putative transgenic plants. **a** T-DNA region of the binary vector plasmid pCAMBIA3301 harboring *AtBG1*. *RB* right border, *LB* left border, P_{35S}, CaMV 35S promoter, P_{Ubi} ubiquitin promoter, *Intron GUS*, GUS coding region containing a catalase intron insertion, *bar* phosphinotricin acetyltransferase gene, *AtBG1*, *Arabidopsis* β-glucosidase gene, T_{35S} CaMV 35S terminator, T_{NOS} *A. tumefaciens nos* gene terminator; T_{Arbcs} *Arbcs* gene terminator. *Arrows* indicate directions of transcription. **b** Herbicide resistance assay. Numbers in lanes (*#1*, *#2*, *#23*, *#24*, *#61*, *#63*) represent transgenic lines selected for this analysis. 0.8 % BASTA® was sprayed onto non-transgenic wild-type control plant (*NT*) and transgenic plants, and the herbicide resistance of the plants was determined 10 days later

Statistical analysis

IBM SPSS statistics 20 software was used for ANOVA to analyze the results of the physiological parameters and ABA concentrations. Significant differences from the control values were determined at $P < 0.05$ or $P < 0.001$ levels. All of the data represented the mean ± SD of at least three independent experiments. The results of NT and HR were not significantly different in all experiments in this study.

Results

Production of transgenic creeping bentgrass

To improve stress tolerance by increasing bioactive ABA levels, the *AtBG1* gene was introduced into creeping bentgrass cv. "Crenshaw" through *Agrobacterium*-mediated transformation. Embryogenic calli derived from mature seeds and the binary vector pCAMBIA3301 harboring the *AtBG1* gene (Fig. 1a) was used for transformation by following a method that we previously established for creeping bentgrass regeneration and transformation (Cho et al. 2011; Han et al. 2009; Kim et al. 2007). Since the binary vector used for the transformation contained the *bar* herbicide resistance gene, putative transgenic creeping bentgrass plants were identified by BASTA resistance assay. The in vitro regenerated putative transgenic lines were grown in a greenhouse for 2 weeks and then sprayed with 0.8 % (v/v) BASTA®. Following herbicide treatment, non-transgenic wild-type control plants (NT) died within 10 days, whereas all of the putative transformants were resistant to herbicide (Fig. 1b). When the *Agrobacterium*-mediated transformation efficiency was calculated as a percentage of herbicide-resistant plants obtained from all infected calli, a transformation efficiency of 5.9 % (43 out of 725) was obtained, which was lower than the previously reported efficiencies (10.7–16.4%) for Crenshaw cultivar (Cho et al. 2011; Han et al. 2009; Kim et al. 2007). These putative transgenic lines were used further for molecular and phenotypic analyses.

Molecular analyses of transgenic plants

To verify the insertion of *bar* and *AtBG1* transgenes, PCR assays on genomic DNA extracted from the leaves of independent transgenic lines were performed. Integration of both transgenes was observed in all herbicide-resistant plants (Supplementary Fig. 1a), whereas no amplified band was observed in the PCR of the total DNA from non-transformed control plant (NT). The integration of foreign DNA into the plant genome was further confirmed by Southern blot hybridization using the *bar*-specific probe (Supplemental Fig. 1b). In the Southern blot analysis of 25 (out of 43) randomly selected transgenic plants, five independent lines of AtBG1 transgenic plants were shown to be successfully produced. No hybridization signal was observed when probing DNA samples derived from non-transformed control plant. However, many of the transgenic lines showed similar band patterns in the blots,

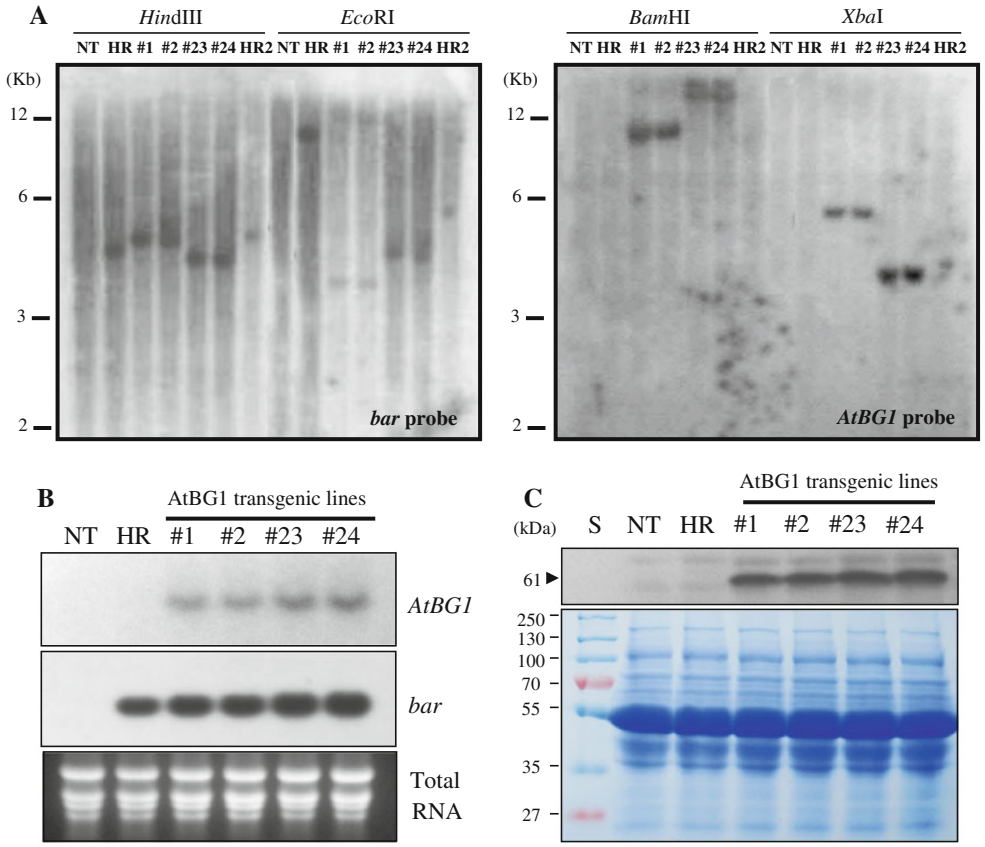

Fig. 2 Molecular analyses of representative transgenic plants. **a** Southern blot analysis of representative transgenic plants. Genomic DNA (15 μg) from each transgenic plant was digested with either *Hin*dIII, *Eco*RI, *Bam*HI or *Xba*I, and then probed with the *bar* or *AtBG1* gene. *NT* non-transgenic control plant, *HR* and *HR2* two herbicide-resistant plants as controls expressing *bar* (i.e., transgenic bentgrass with pCAMBIA3301). *Numbers* in *lanes* represent transgenic lines selected for analysis. **b** Northern blot analysis. Total RNA was isolated from the leaves of transgenic plants and *bar* or *AtBG1* was used as a probe. Total RNA was shown as a loading control. **c** Western blot analysis. Crude extracts of leaves were isolated and loaded onto 10 % SDS-PAGE. An HA-specific monoclonal antibody was used for the detection of HA-tagged AtBG1. SDS-PAGE gel was shown as a loading control. *S* standard protein markers

suggesting that they might have originated from the same callus. In a subsequent phenotypic investigation, transgenic lines with single transgene integration were selected for further analysis, which were the #1, #2, #23, and #24 lines.

Using the selected lines, Southern blot hybridizations were conducted with *bar*- and *AtBG1*-specific probes (Fig. 2a). The results showed the single transgene integration in the lines. The #1 and #2 lines showed the same band pattern in the blots, suggesting that they were the transgenic lines originated from the same callus. In addition, the #23 and #24 lines were also confirmed as the same lines. In contrast, bands in transgenic plants with pCAMBIA3301 (i.e., herbicide-resistant bentgrass; HR) were only observed with the *bar*-specific probe. The expression of both transgenes was then analyzed by Northern blot analysis using *bar* and *AtBG1* probes (Fig. 2b). While all of the selected transgenic lines expressed the integrated transgenes, no hybridization was detected in the non-transgenic control plant, and HR plant showed only the expression of *bar*. Western blot analysis with the HA-specific monoclonal antibody further confirmed the expression of HA-tagged AtBG1 proteins in transgenic lines (Fig. 2c). Collectively, we obtained independent transgenic lines with single transgene integration showing similar protein expression levels.

Transgenic plants with AtBG1 displayed dwarf phenotypes with retarded growth

The independent AtBG1 transgenic lines were then grown in a greenhouse and phenotypic analyses were performed by comparing the transgenic plants with non-transgenic control plant (NT) and transgenic plant with pCAMBIA3301 vector (HR). Strikingly, the AtBG1 transgenic creeping bentgrass plants displayed dwarf phenotypes during growth in the greenhouse (Supplemental Fig. 2 and Fig. 3a). The leaf lengths of 4 week-grown NT and HR plants were approximately 12 cm, whereas those of the

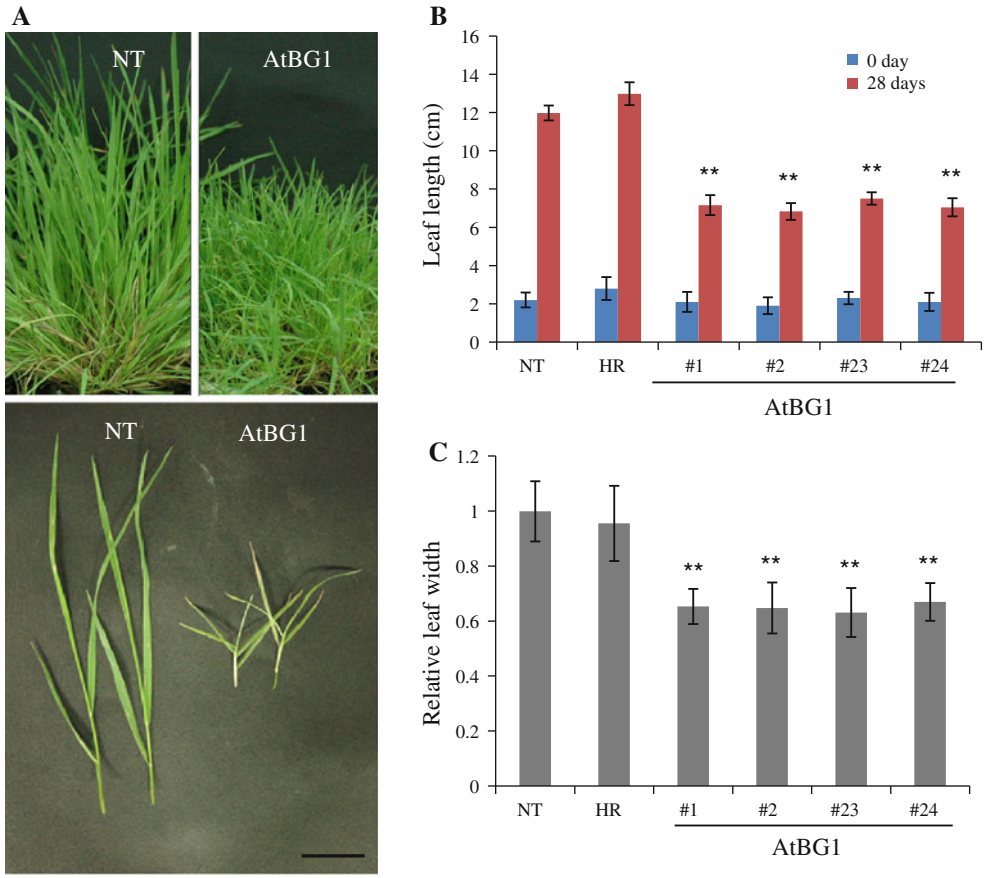

Fig. 3 Phenotypic analyses of representative transgenic plants. **a** Fully-grown AtBG1 transgenic plants and non-transgenic control plants (*NT*). The plants grown to similar stages were trimmed to the same heights, and then the trimmed plants were further grown for 4 weeks and the pictures were taken. *HR* herbicide-resistant creeping bentgrass as a control (i.e., transgenic bentgrass with pCAMBIA3301). **b**, **c** Measurements of leaf lengths and relative leaf widths. Leaf lengths were measured from the second leaf of the first stoloniferous plant, and leaf widths were measured on the widest part from the second leaf. The relative leaf width was calculated by setting the leaf width of non-transgenic bentgrass (NT) to 1. Data represent mean ± SD of three independent measurements. Statistically significant changes compared with NT or HR are indicated by *asterisks* when $P < 0.001$ (**$P < 0.001$ vs. NT or HR)

AtBG1 transgenic plants were about 7 cm (Fig. 3b). In addition, the leaf widths of the AtBG1 transgenic plants were also significantly reduced (~35 %) when compared with NT or the HR plant (Fig. 3c). Both independent AtBG1 transgenic lines, #1/#2 and #23/#24, showed very similar dwarf phenotypes, suggesting that the dwarf phenotype was due to the overexpression of AtBG1 in creeping bentgrass.

To directly determine whether the dwarf phenotypes were due to the function of AtBG1 but not the effects of T-DNA insertion, inverse PCR (I-PCR) analyses were further performed to investigate the insertion sites of T-DNA in two selected independent transgenic lines, #2 and #23 (Supplemental Fig. 3 and Supplemental Fig. 4). Based on the sequencing analysis of DNA regions upstream or downstream to the T-DNA insertion, we found different insertions of T-DNA in #2 and #23 transgenic lines and the T-DNA was inserted into non-coding regions in the two lines. Therefore, the T-DNA insertion might not affect the growth and development of creeping bentgrass, confirming that the dwarf phenotype in transgenic creeping bentgrass was caused by the overexpression of *AtBG1*.

AtBG1 was reported to produce active ABA by hydrolyzing glucose-conjugated inactive ABA, and thus ABA levels were increased in AtBG1-overexpressing *Arabidopsis* plants (Lee et al. 2006). Since it has been known that high levels of ABA in plants have inhibitory effects on plant growth, we further investigated the leaf growth of the AtBG1 transgenic plants in a time-dependent manner to confirm that the dwarf phenotype was caused by growth retardation. In the NT and HR creeping bentgrass plants, the leaf lengths gradually increased with a maximum leaf growth rate at 20 days after growth (Fig. 4). Under our growth conditions, the leaf growth rates increased up to 20 days and then decreased. In the AtBG1 transgenic plants, the changes in the leaf growth rates were very similar to the NT and HR. However, the magnitude of the growth rates were significantly reduced in the AtBG1

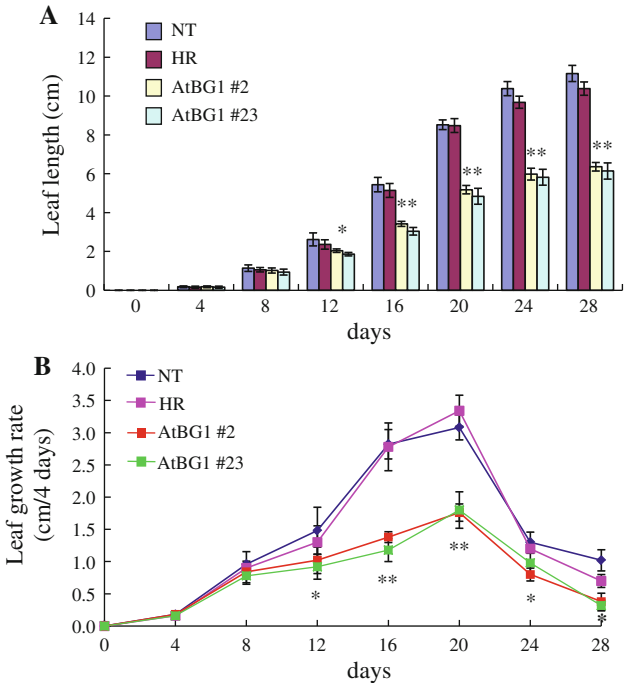

Fig. 4 Analysis of leaf growth rates of the AtBG1 transgenic plants. **a** Growth-time dependent measurements of leaf growth. After the plants grown at similar stages were trimmed to the same heights, the leaf lengths were measured at 4-day intervals. **b** Measurements of leaf growth rates. Leaf growth rates (cm/4 days) were calculated from ((the leaf length of current measurement) − (the leaf length of previous measurement)) at the intervals of 4 days. Data represent mean ± SD of three independent measurements. Statistically significant changes compared with NT or HR are indicated by *single asterisk* when $P < 0.05$ or by *double asterisk* when $P < 0.001$ (*$P < 0.05$, **$P < 0.001$ vs. NT or HR)

transgenic plants compared to the NT and HR plants (Fig. 4b). Therefore, these results suggest that the transgenic plants overexpressing AtBG1 display a dwarf phenotype due to reduced growth rates.

Overexpression of AtBG1 increased bioactive ABA levels and enhanced drought tolerance in creeping bentgrass

AtBG1 was shown to hydrolyze ABA-GE to bioactive ABA, which is believed to be a quick release mechanism for ABA (Schroeder and Nambara 2006). Thus, it was speculated that the overexpression of AtBG1 might increase the bioactive ABA levels in plants. Actually, the dwarf phenotype of the AtBG1 transgenic creeping bentgrass plants was also hypothesized to be due to the increased ABA levels. Therefore, we measured endogenous ABA levels in the AtBG1 transgenic creeping bentgrass plants including NT and HR plants. Under non-stressed condition, the ABA concentrations in the NT and HR plants were very low (i.e., basal ABA levels; ~14 pmol/g FW), whereas those in the AtBG1 transgenic plants were approximately 15 times higher (~210 pmol/g FW) than the NT or HR plants (Fig. 5a). Under drought-stressed condition, ABA levels were elevated in response to drought stress in all tested plants (Fig. 5b). The ABA levels were highly increased in NT and HR plants (approximately 25 times; 14 vs. 350 pmol/g FW), as expected. In the AtBG1 transgenic plants, the ABA content was increased approximately five to six times higher than

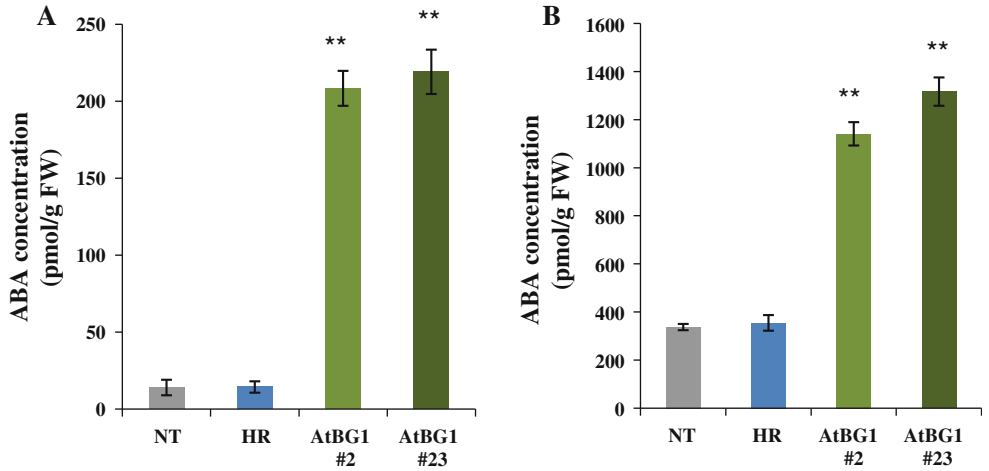

Fig. 5 Analysis of endogenous ABA levels in the AtBG1 transgenic plants. **a** Measurements of ABA levels under non-stressed condition. All tested plants were planted on 12 cm diameter pots and cultured for 4 weeks. 0.1 g of fresh weight of leaves from the 4-week-grown plants was used for this assay. **b** Measurements of ABA levels under drought-stressed condition. Drought treatment was carried out by air-drying detached leaves at room temperature for 2 h. The dehydrated samples were then incubated in the dark for 4 h at room temperature before the measurement. Data represent mean ± SD of three independent measurements (**$P < 0.001$ vs. NT or HR)

the transgenic plants grown in the non-stressed condition. When the ABA content under the dehydrated condition was compared among the tested plants, the AtBG1 transgenic plants were shown to contain ABA levels that were more than three times higher than the NT or HR plant. In addition, the ABA levels in the AtBG1 transgenic plants under the non-stressed condition were close to two-thirds of the ABA content in NT or HR plant under the drought-stressed condition (Fig. 5). These results demonstrated that the concentration of the active ABA form in the AtBG1 transgenic plants was greatly increased under both non-stressed and drought-stressed conditions when compared to the wild-type creeping bentgrass plant.

Since the AtBG1 transgenic plants contained highly elevated levels of active ABA, we further investigated the tolerance to drought stress. Drought tolerance was investigated by withholding irrigation for 4 weeks, and the results showed that the AtBG1 transgenic plants were significantly tolerant to drought stress (Fig. 6a). The water content of NT or HR plant was rapidly lowered during the drought treatment, whereas that of the AtBG1 transgenic plants was slowly reduced (Fig. 6b). The water content of NT or HR plant was decreased to below 20 % after 28 days of drought treatment, while that of the AtBG1 transgenic plants was more than 70 %. Under our experimental conditions, 4 weeks after terminating irrigation, only the AtBG1 transgenic plants had recovered by re-irrigation after 2 weeks, while the NT and HR plants did not recover (Fig. 6a). These results suggest that higher ABA levels in the AtBG1 transgenic plants could enhance drought stress tolerance.

Collectively, the present study demonstrates that the overexpression of AtBG1 induces the accumulation of higher ABA levels in plants, which results in retarded growth displaying dwarf phenotype and also enhances the survival ability in water-limiting environments. Therefore, genetically engineered dwarf creeping bentgrass with drought resistance has been developed by introducing *AtBG1*, and our results suggest that manipulating ABA levels by AtBG1 may provide an effective means to increase drought stress tolerance in other plants.

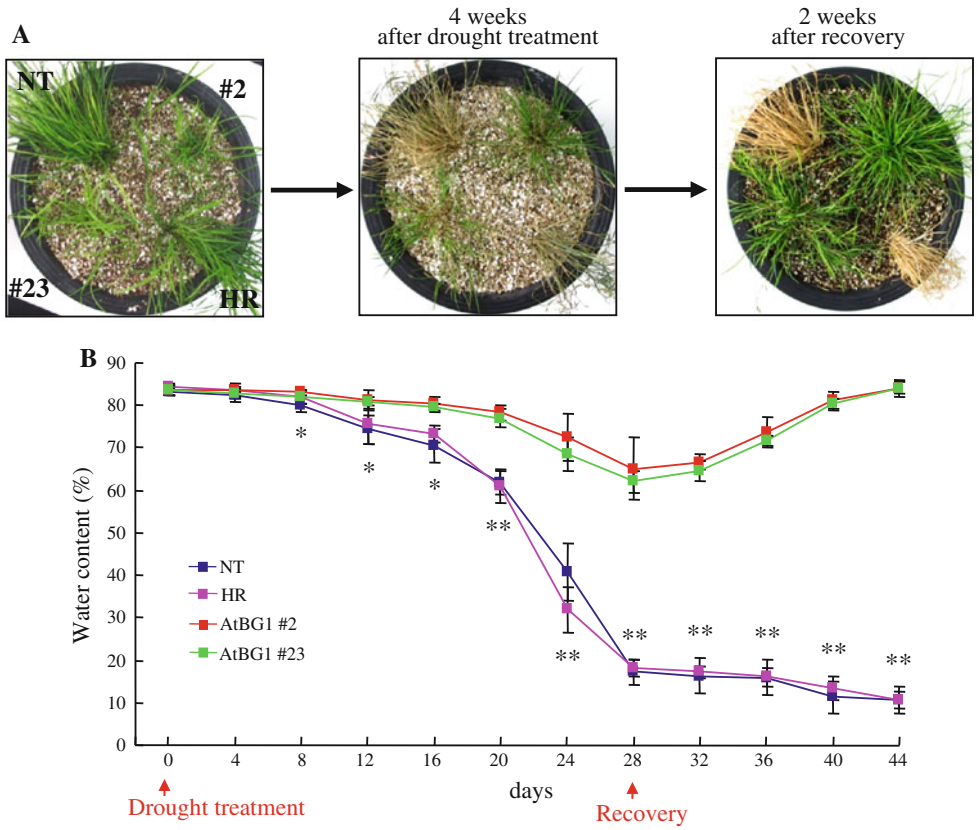

Fig. 6 Drought tolerance of AtBG1 transgenic plants. **a** Drought tolerance assay. Plants grown at similar stages in 25 cm diameter pots were subjected to drought stress. The pots were fully irrigated before the start of the drought tolerance assay, and then subjected to drought stress by terminating irrigation. After 4 weeks of drought treatment, the plants were re-irrigated and photographed 2 weeks after re-irrigation. **b** Measurements of water content. The leaves were harvested every 4 days after irrigation was stopped, and the fresh weight and dry weight were measured to calculate the water content. Data represent mean ± SD of three independent measurements (*$P < 0.05$, **$P < 0.001$ vs. NT or HR)

Discussion

In the present study, we successfully produced dwarf creeping bentgrass plants with drought tolerance, as well as herbicide resistance, by introducing pCAMBIA3301 harboring *AtBG1* under the control of the ubiquitin promoter. These dwarf and drought tolerance phenotypes could be explained by the function of AtBG1, which hydrolyzes biologically inactive ABA-GE to bioactive ABA. The results of the ABA quantification revealed an increase in the ABA levels in the AtBG1 transgenic plants (Fig. 5). The active ABA levels of the AtBG1 transgenic creeping bentgrass plants under non-stressed condition were approximately 15 times higher than the wild-type bentgrass plant. This result might be due to constitutive overexpression of AtBG1 by the ubiquitin promoter in the transgenic plants. In addition, the ABA levels were also five to six times higher in the AtBG1 transgenic plants under dehydration-stressed conditions when compared to the wild-type plant. This elevated ABA level might not be explained by the constitutive overexpression of AtBG1, but it could be explained by the characteristic of AtBG1; the enzymatic activity of AtBG1 in ABA-GE hydrolysis was shown to be activated through its stress-induced polymerization (Lee et al. 2006). This means that drought treatment induces the polymerization of AtBG1 proteins and activates its glucosidase activity. Since there are more AtBG1 proteins in AtBG1 transgenic plants than wild-type plants, the enzymatic activation of AtBG1 via stress-induced polymerization might be higher in the AtBG1 transgenic plants, resulting in elevated ABA level under drought condition. Therefore, the ABA levels in the AtBG1 transgenic plants were higher under both non-stressed and dehydration-stressed conditions than the wild-type bentgrass plant, which made the AtBG1 transgenic plants more tolerant to drought.

Previously, engineering plants with elevated ABA levels and subsequent stress tolerance were developed mostly using enzymes involved in ABA biosynthesis, including *NCED* and *ZEP*. As examples, ectopic expression of a tomato NCED gene (*LeNCED1*) has been reported to induce over-production of ABA, and transgenic plants overexpressing *LeNCED1* exhibited severe phenotypes, such as increased photobleaching in young seedlings, substantially reduced chlorophyll, and greatly reduced growth (Thompson et al. 2000; Tung et al. 2008). The expression of an *Arabidopsis NCED* gene (*AtNCED3*) was also reported to improve drought tolerance with the accumulation of endogenous ABA (Iuchi et al. 2001). In addition, overexpression of *ZEP* was reported to enhance tolerance to osmotic stress with increased ABA levels (Park et al. 2008). However, it has been suggested that engineering plants for increased ABA levels using ABA biosynthetic genes might be difficult because of the feedback regulation in controlling ABA metabolism (Verslues and Zhu 2007). For e.g., high levels of *AtNCED3* overexpression led to only relatively small increases or no effect on ABA content under stress conditions (Priest et al. 2006), but much larger increases in phaseic acid, the principle ABA catabolite (Qin and Zeevaart 2002). Furthermore, the ABA biosynthetic pathway shares the intermediates required in other metabolic pathways and the overexpression of ABA biosynthetic genes might exhaust these intermediates (Taylor et al. 2005). Therefore, we used the ABA-GE hydrolyzing enzyme, AtBG1, for the first time to engineer crops with elevated ABA levels. The previous study of *AtBG1* suggested that ABA conjugation and deconjugation are dynamic processes and these processes play a significant role in controlling the levels of biologically active ABA both under unstressed condition and under stress when the amount of free ABA increases dramatically (Lee et al. 2006). The present study demonstrated that overexpression of AtBG1 increased the endogenous ABA levels under both unstressed and stress conditions, which exhibited severe phenotypes, including a dwarf phenotype, in creeping bentgrass. Therefore, the expression of AtBG1 in plants might be more effective in manipulating ABA levels than that of ABA biosynthetic genes, providing an effective means to develop crops with enhanced water-use efficiency and improved resistance to abiotic stresses, such as drought.

During the transformation, we interestingly found that the transformation efficiency with pCAMBIA3301 harboring *AtBG1* was lower (5.9 %) than the transformation efficiencies (10.7–16.4 %) in our previous reports (Cho et al. 2011; Han et al. 2009; Kim et al. 2007). If we considered the number of independent lines identified via Southern blots, the transformation efficiency was about 1.19 % (based on five independent lines in 25 transgenic plants, 8.6 independent lines in 43 transgenic plants from 725 infected calli were expected). Compared to the previously reported transformation efficiency of creeping bentgrass cv. Crenshow (7.55–9.50 %; Kim et al. 2007), 1.19 % transformation efficiency for AtBG1 transformation was quite low. Since the transformation procedures used in the present study were essentially the same as those in the previous reports, the low transformation efficiency might also be due to the increased ABA levels in embryogenic calli. This result is consistent with the previous result of tomato transformation, in which relatively few overexpressing primary transformants with the *sp::LeNCED1* transgene were recovered compared to the number typically obtained with unrelated gene constructs (Thompson et al. 2000; Tung et al. 2008). It was suggested that transgenic lines with the transgene and potentially higher ABA accumulation were not recovered from tissue

culture because of deleterious effects on growth. In the present study, we also observed relatively low transformation efficiency with AtBG1 when compared to the transformation efficiencies that we obtained previously.

The AtBG1 transgenic creeping bentgrass developed in the present study have beneficial traits for the turfgrasses of golf courses, which include herbicide resistance, drought tolerance and dwarf phenotypes. Herbicide resistance is useful for controlling unwanted weeds, which allows for easier maintenance of golf courses and lawns, and a decrease in the number and amount of pollution-generating agrochemicals needed. Creeping bentgrass with drought tolerance and reduced growth rates would be a demanded cultivar in breeding programs, because creeping bentgrass often requires daily irrigation and frequent mowing due to its intolerance to drought stress and vigorous growth. Therefore, the herbicide-resistant, drought-tolerant, and dwarf creeping bentgrass reported herein offers great economic and environmental advantages over wild-type creeping bentgrass.

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