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Research Article

Enhanced drought tolerance by expression of *hvDhn5* gene in poplar

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Abstract We transferred *Dhn5* (dehydrin5) gene from barley to poplar to determine the effect of its expression on the transgenic poplars. The results from northern blot analysis showed that the expression level of gene varied among the transgenic lines. During their culture on tissue culture media, the transgenic poplars formed vigorous growing callus in the presence of 5% PEG. When the transgenic poplars were growing in pots and witheld watering, they stayed much healthier than nontransgenic poplars. The transgenic poplars showed higher rates of photosynthetic rates, stomatal conductance and evaporation rates under the drought stress, although there was no significant difference in soil water content within the treatments. The relative electrical conductivity of the transgenic poplars after 20% PEG treatment was lower than that of nontransgenic poplars. The results provide evidence that the expression of *hvDhn5* gene conferred drought tolerance in the transgenic poplars.

Keywords Dehydrin5, Drought tolerance, Photosynthetic rates, Relative electrical conductivity, Transgenic poplar

Introduction

Salt, drought and freezing stresses are the most critical abiotic stresses that affect productivity of annual crop species as well as perennial trees (Caruso et al. 2002). Recently rapid desertification has resulted in increased interest in the development of crop varieties that could tolerate the various environmental stresses. However, different plants adapt to their environment by different mechanisms. Therefore, it has been very difficult to select or develop varieties which withstand various environmental stresses via conventional breeding. The stacking of those traits into a selected variety requires cross fertility between the organisms that carry traits of interest (Flowers and Yeo 1995). This barrier could be overcome by genetic engineering (Singsit et al. 1997). It is now possible to selectively transfer only the traits of interest into a new plant variety so that we could avoid to transfer the unwanted portion of DNA in the donor genome (Cushman and Bohnert 2000).

A number of promising genes for salt and drought tolerance have been reported. Among them are LEA (late embryogenesis-abundant protein) genes (Brini et al. 2007b). Dehydrin belongs to LEA II group protein and known to be induced by environmental stresses including drought and cold temperature. Dehyrin is thought to play a role as a stabilizer of cellular macromolecules (Close 1996, 1997; Ingram & Bartels 1994). For example, transgenic plants expressing LEA genes from barley and corn showed increased tolerance against drought and salt stresses (Xu et al. 1996; Figueras et al. 2004). So far 13 dehydrin (*Dhn* 1 to 13) genes from barley have been reported (Rodriguez et al. 2005). Among them, *Dhn5* is known to be induced by low temperature stress and involved in freezing tolerance by protecting meristematic cells from losing water (Zhu et al. 2000). Han and Hwang (2003) transferred the barley *Dhn5* gene to *Artemisia adamsii* and reported that the calli from transgenic plants grew much better than those from nontransgenic plants in the presence of 20% PEG. Similarly, transgenic *Arabidopsis* carrying wheat *Dhn5* gene displayed enhanced tolerance against salt and osmotic stresses (Brini et al. 2007a). Many other cases of transgenic plants expressing LEA/Dhn proteins with increased tolerance against salt, drought and cold stresses have been reported (Xu et al. 1996; Babu et al. 2004; Sivamani et al. 2000; Honjoh et al. 1999).

We were interested in confirming whether the gene could confer poplar, a woody species, tolerance against such stresses. Therefore, in the present study, we introduced barley *Dhn5* gene to poplar and examined the phenotypes of the transgenic poplars under drought stress.

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Materials and methods

Gene cloning and vector construction

Barley *Dhn5* gene was PCR cloned from barley genomic DNA using published sequence (GenBank accession No. M95810). A primer set was designed to amplify the gene: Forward: ggatcctaccctcga acggcctataa, and Reverse: gagctc tgaatgtgcatacggtgga. An enzyme site (*Bam*H1 in F primer and *Sac*1 in R primer, respectively) was inserted to each primer to ease cloning process. The amplified PCR product was inserted into pBI121 after GUS gene was removed by *Bam*H1 and *Sac*1 digestion and transferred to an *Agrobacterium tumefaciens* vector, the strain GV3101.

Poplar transformation and regeneration

The chimeric *p35S-hvDhn5* gene was introduced to poplar by *A. tumefaciens* mediated transformation (Fillatti et al. 1987). Transformed cells were selected on MS medium (Murashige and Skoog 1962) containing 1.0 mg/L 2.4-D, 0.1 mg/L BAP, 0.01 mg/L NAA, 500 mg/L cefotaxime plus 50 mg/L kanamycin. Shoots were regenerated on WPM (Lloyd and McCown 1981) containing 1.0 mg/L zeatin, 0.1 mg/L BA, 0.01 mg/L NAA and 50 mg/L kanamycin.

Molecular analysis

Genomic DNA was extracted from the leaves of poplar plants using a MagExtractor-Plant Genome kit (Toyobo, Osaka, Japan). Ten μg of the genomic DNA were digested with a restriction enzyme *Pst* 1 overnight, run on 1% agarose gel and then transferred to Hybond-XL nylon membrane by capillary transfer method (Southern 1975). The full-length *hvDhn5* coding sequence labelled with ^{32}P-dCTP was used as probe for hybridization for 12 h. The membrane was washed in 2 × SSC and 0.1% SDS (50℃) for 10 min and in 0.2 × SSC and 0.1% SDS (50℃) for 30 min followed by exposing to an X-ray film at -70℃.

For northern blot, the total RNA was isolated from young leaves using TRI Reagent (Molecular Research Center Inc., Cincinnati, OH, USA). Ten μg of the RNA were run on 1.2% formaldehyde agarose gel and then transferred to Hybond-XL nylon membrane. The methods for probe preparation and hybridization were the same as in Southern hybridization.

Drought tolerance assays

For drought tolerance assay, four different transgenic clones (*Dhn5*-1, 5-3, 5-9, and 5-10) showing different expression levels were used. To determine tolerance of the transgenic lines to drought stress, both *in vitro* and greenhouse pot trials were performed. For *in vitro* drought tolerance assay, leaf fragments (0.5 cm × 1.0 cm) were cultured on callus inducing media (MS with 1.0 mg/L 2.4-D and 0.1 mg/L BAP) containing various levels (0, 5, 10%) of polyethylene glycol (PEG). Fresh weight growth of the calli was determined after 5 weeks growth on culture medium. Drought tolerance of the two transgenic lines (*Dhn5*-1 and *Dhn 5*-10) was assessed in a pot trial in the greenhouse. The plants were transferred to plastic pots filled with sand soil and fully watered. After 28 days, transgenic and nontransgenic poplars were subjected to drought treatment by withholding irrigation for 7 days. Reference plants were irrigated at 3 to 4 day interval. The photosynthesis of the poplars was compared when damage symptoms were visible. A portable photosynthesis system (model LI-6400, LI-COR, Lincoln, NE) was used to determine photosynthetic rates. After photosynthesis measurement, soil water content at the end of 7 days was measured.

Ion leakage analysis

Ion leakage of the leaves was measured by relative electrical conductivity (REC) as described by Wu et al. (2008) with some modifications. Leaf tissue was cut into segments of 1 cm^2 with cork borer (No. 6). Five leaf segments were then drought stress for 48 hrs with 20% PEG solution at room temperature (25±2℃). After the stress, the leaf segments were washed quickly five times with distilled deionized water and then put into 15 ml of distilled deionized water in falcon tube (50 ml) and shaked using a rotary shaker (170 rpm) at room temperature (25±2℃). Electrical conductivity (EC) was measured at 1 hr later using an ion conductivity meter (Mettler co.) (E_1). Then, the tubes containing the segments were autoclaved for 20 min at 121℃ and the conductivity of the effused was measured after cooling it to room temperature (E_2). Relative electrical conductivity was measured by the formula $E_1 / E_2 \times 100$.

Results and discussion

Development and molecular analyses of transgenic poplars

Transgenic calli were first screened on callus inducing medium in the presence of 50 mg/l kanamycin. Shoots were also regenerated from the calli in the presence of 50 mg/L kanamycin. Southern blot analysis using barley *Dhn5* gene

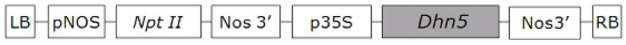


Fig. 1 Schematic illustration of a plant expression vector carrying *p35S-hvDhn5* gene (*Dhn5*: barley dehydrin5 gene, p35S: CaMV35S promoter, pNOS: nopaline synthase promoter, *npt II*: neomycin phosphotransferase II, NOS3': nopaline synthase terminator)

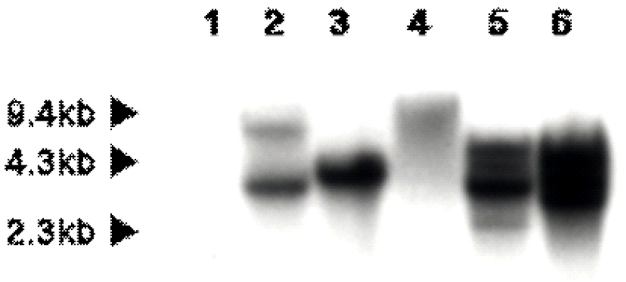


Fig. 2 Southern blot analysis of poplars transformed by *p35S-hvDhn5*. Genomic DNA (10μg) from regenerated poplars was digested with *Pst* 1 and separated by electrophoresis in an 1.0% agarose gel. The gel was blotted onto nylon membrane, and hybridized with ^{32}P-la beledfull-length *hvDhn5* gene. 1: nontransgenic poplar and 2 to 6: transgenic poplars

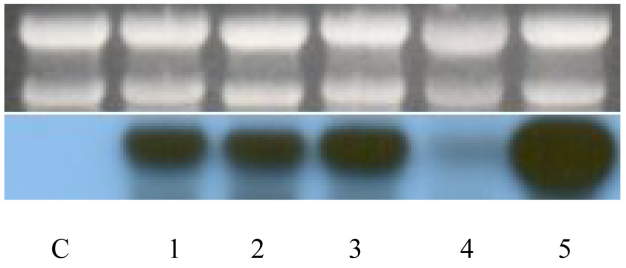


Fig. 3 Expression of *p35S-hvDhn5* gene revealed by Northern blot analysis. 10 μg of total RNA extracted from leaf tissues were resolved on formaldehyde agarose gel and blotted onto nylon membrane. Hybridization was done with 32P-labeled probe of full length *hv Dhn5* gene. The top is EtBr-stained gel showing rRNA bands. C: nontransgenic poplar and 1 to 5: transgenic poplars

as a probe revealed that out of 5 transgenic poplars examined, two contained a single copy transgene and three contained multiple copies (Fig. 2). Northern blot using barley *Dhn5* gene showed that expression level varied much up to 10 fold difference among transgenic lines (Fig. 3). The difference in the transcription level among transgenic lines may be due to different copy number or different integration sites in the genome (Cooley et al. 1995; Sarria et al. 2000).

Drought tolerance test using test tube plants

To determine tolerance of the *hvDhn5* transgenic poplars to drought stress, calli were induced from the transgenic lines on callus inducing (and growing) medium containing

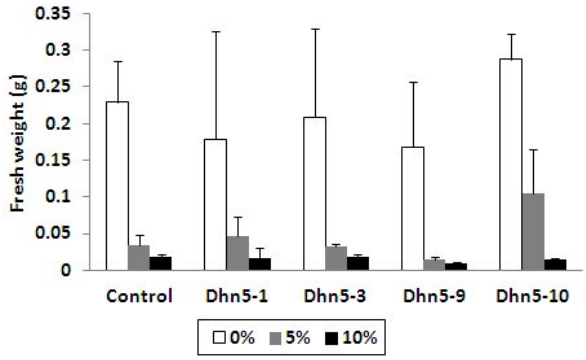


Fig. 4 Fresh weight of callus of poplar with a barley *Dhn5* gene grown in media supplemented with PEG. Control is the nontransgenic poplars; *Dhn5*-1, *Dhn5*-3, *Dhn5*-9, and *Dhn5*-10 are the transgenic poplars with the *hvDhn5* gene. All the plants were treated with PEG for 28 days. Vertical bars represent standard errors (n=3 per experiment)

0, 5, 10% PEG. Although all the lines tested formed vigorously growing callus in the absence of PEG, they all showed growth suppression in the presence of PEG. However, transgenic lines showed less damage by PEG imposed stress than untransformed control. In the presence of 5% PEG nontransgenic poplars attained only 15% of callus growth (in fresh weight) that could be produced in the absence of PEG. In contrast, transgenic lines showed 26 and 37% of callus growth otherwise obtained in the absence of PEG. However, at 10% PEG, all the lines showed growth suppression and did not show any difference in the growth among the lines (Fig. 4). Transgenic *Artemisia* carrying the same *Dhn5* gene was reported to tolerate up to 20% PEG. The difference may be genetic nature of the plant materials. *Artemisia adamsii* is native to Gobi desert and thus thought to be highly tolerant to stress (Han and Hwang 2003). There might be other factors that protect *Artemisia* from drought or osmotic stresses.

Drought tolerance test using pot plants

hvDhn5 transgenic poplars withheld watering for a week did not show any wilting symptoms while nontransgenic poplars suffered from severe wilting damage (Fig. 5). There was no difference in photosynthetic rate between transgenic and nontransgenic poplars in normal conditions. However, at the time of wilting symptoms developed, the photosynthetic rate of nontransgenic poplars rapidly dropped to 33. 7% of that in normal condition. In contrast, the transgenic lines *Dhn5*-1 and *Dhn5*-10 maintained the rates up to 84.6 and 92.7% of normal condition, respectively (Fig. 6-A).

Soil water content was also determined by the time when

(A) (B) (C)

Fig. 5 Growth phenotypes of nontransgenic poplar vs. transgenic poplars over expressing *hvDhn5* gene after 7 days of withholding watering; (A) nontransgenic poplar, (B) and (C) transgenic poplars

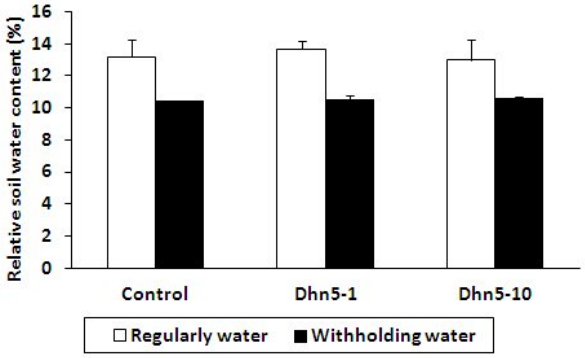


Fig. 7 Relative soil water content after 7 days of withholding watering. Control is the nontransgenic poplars; *Dhn5*-1 and *Dhn5*-10 are the transgenic poplars with the *hvDhn5* gene. All the plants were treated for 7 days. Vertical bars represent standard errors (n=3 per experiment)

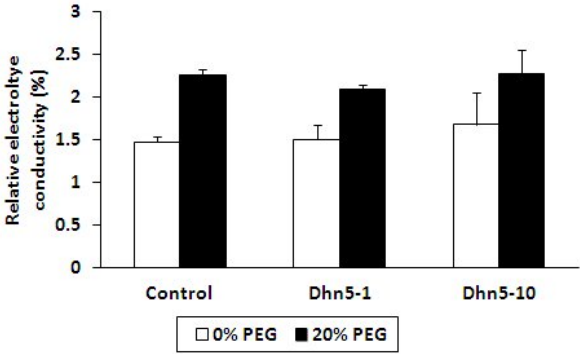
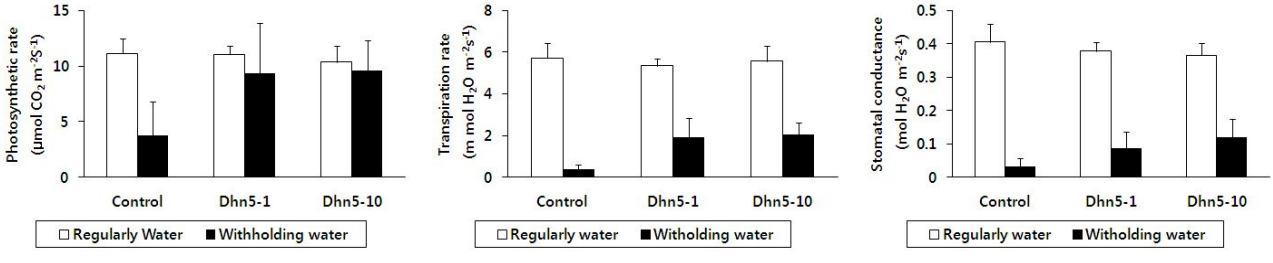


Fig. 8 The relative electrical conductivity in the leaf discs of nontransgenic and transgenic poplars after 20% PEG treatment for 48 hrs. Vertical bars represent standard errors (n=3 per experiment)

photosynthetic rates were measured. There was no difference in soil water content between the pots with transgenic lines and those with nontransgenic poplars (Fig. 7). Both drought and salt stress are known to cause the reduction in photosynthetic rates (Greenway and Munns 1980). When plants are under drought stress, they close stomata and thereby lowering CO_2 level in the mesophyll cells and reducing photosynthetic activity (Farquhar et al. 1987; Gimenez et al. 1992). Oh et al. (2005) also demonstrated that drought stress reduced plant growth and thus productivity of aspen plants by decreasing photosynthesis. Thus, it is critical to maintain photosynthetic structures and photosynthetic activity under drought stress for plant productivity (Tunnacliffe and Wise 2007).

Changes in stomatal conductivity under drought stress appeared to be similar to those in photosynthesis. Whereas nontransgenic poplars under drought stress displayed 8.2% activity of untreated poplars, the two transgenic lines were shown to maintain 23.3% and 32.5%, respectively of untreated poplars under the same conditions (Fig. 6-B). These results suggest that stomata closure play a role in the defense against drought stress and the transgenic lines seem to have better capacity to control stomatal closure. Similar observation was also made by other workers (Chaves et al. 2003).

As for changes in transpiration rate under drought stress, nontransgenic poplars showed markedly 93.2% decrease



(A) (B) (C)

Fig. 6 Photosynthetic rate (A), transpiration rate (B) and stomatal conductance (C) of *p35S-hvDhn5* transgenic poplars after 7 days of withholding watering. Control is the nontransgenic poplars; *Dhn5*-1 and *Dhn5*-10 are the transgenic poplars with the *hvDhn5* gene. All the plants were treated for 7 days. Vertical bars represent standard errors (n=3 per experiment)

when compared to untreated poplars. In contrast, transgenic lines showed merely decreased rates of 64.3% and 62.8%, respectively when compared to untreated poplars (Fig. 6-C). Thus, the transgenic lines have higher activities in photosynthesis, stomatal conductivity, and transpiration than nontransgenic poplars. Therefore, overall, transgenic lines were more tolerant and thus showed less damage symptoms.

Ion leakage test

Figure 8 shows the relative ion conductivity of the solution exuded from leaf disks after 20% PEG treatment. There was no significant difference between transgenic and nontransgenic poplars in the absence of PEG. However, when treated with PEG, the relative ion conductivity was significantly lower in the solution from transgenic lines than that from nontransgenic poplars. Whereas the ion conductivity of nontransgenic poplars increased 153.3% by PEG treatment, those of transgenic lines increased to 138.4% and 136.5%, respectively. The result is consistent with the previous reports showing lower ion conductivity in both transgenic tobacco and rice plants expressing LEA or *HVA1* gene (Wang et al. 2006; Liu et al. 2009). Wang et al. (2006) conjectured that the low ion conductivity might be due to less damage of the transgenic plants since the LEA proteins protected the membrane under drought stress. Similarly, LEA proteins act as a stabilizer of cellular molecules and thus protect cellular components under the stress (Tunnacliffe and Wise 2007; Bajji et al. 2002). Therefore, the transgenic poplar lines are more tolerant to drought stress than nontransgneic poplars due to the expression of *hvDhn5* gene.

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