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Transformation of tomato cultivar 'Pusa Ruby' with *bspA* gene from *Populus tremula* for drought tolerance

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Abstract

Boiling stable proteins are known to play a role in desiccation tolerance against water stress by protecting proteins in membranes and cytosol. A novel 66 kDa boiling stable protein was highly expressed in *Populus tremula* in response to gradual water stress. The plasmid carrying cDNA encoding this protein was transferred to tomato (*Lycopersicon esculentum* cultivar 'Pusa Ruby') through *Agrobacterium tumefaciens*. Initially, hygromycin resistance was used for selection of transient gene expression. Stable integration of the T-DNA into nuclear genome was confirmed by PCR and PCR-Southern hybridization. Northern blot analysis detected the different levels of transcripts of *bspA* gene in individual transgenic lines. Drought tolerance of putative transgenic lines was assayed by PEG test, biomass analysis, proline estimation and electrolyte leakage measurement. All of them showed slightly increased tolerance of transgenic plants to water stress compared to non-transgenics.

Abbreviations: bspA – boiling stable protein gene of aspen; EtBr – ethidium bromide; *hpt* – hygromycin phosphotransferase gene; Hyg – hygromycin; IAA – indole-3-acetic acid; LB – Luria-Bertani; LEA – late embryogenesis abundant; MS – Murashige and Skoog; PCR – polymerase chain reaction; PEG – polyethylene glycol

Introduction

Plant biotechnology techniques have been successfully applied for developing biotic stress resistant plants through genetic transformations but it is yet to do so for abiotic stress resistance such as drought, salinity, high temperature, chemical toxicity and oxidative stresses. The reason being that abiotic stress is multigenic and depends on a series of events as well as 'protective mechanisms' acting together (Altman, 2003). It is difficult to engineer the multigenic traits of resistance to abiotic stresses compared to monogenic of resistance to pests and herbicides. However, drought and salinity are the most serious threats to agriculture and are far more important globally (Altman, 2003). Water stress is a major harmful factor in arid and semi-arid regions worldwide. It results in the expression of many new genes and the synthesis of resultant new proteins (Gòmez et al., 1988; Mundy and Chua, 1988; Close et al., 1989; Piatkowski et al., 1990; Vierling and Kimpel, 1992). Some drought induced proteins like dehydrins are highly hydrophilic and remain stable even after boiling (Close et al., 1989; Purty et al., 2005), a characteristic that has been termed 'boiling stability' (Jacobsen and Shaw, 1989). The induction and conservation of dehydrins by desiccation, in several plant species, have led to the suggestion that these proteins protect membranal and other cell proteins

during water loss (Baker et al., 1988; Close et al., 1989; Close and Chandler, 1990; Skriver and Mundy, 1990; Pelah et al., 1995, 1997; Caruso et al., 2002). Using a transgenic approach, Xu et al. (1996) provided direct evidence that LEA proteins play an important role in protecting plants against water or salt stress.

Tomato is a major agricultural commodity and, therefore, water stress tolerance will facilitate its production even in the drier regions. Tomatoes have been transformed using different genes, e.g. anti-ripening (Hamilton et al., 1990, 1995; Klee et al., 1991; Theologis, 1992; Picton et al., 1993; Theologis et al., 1993; Redenbaugh et al., 1995; Reed et al., 1996), insect (Fischhoff et al., 1987) and herbicide tolerance (Fillatti et al., 1987a, b), virus resistance (Nelson et al., 1988; Kim et al., 1994; Witham et al., 1996), male sterility (Mariani et al., 1992) and salt tolerance (Gisbert et al., 2000; Jia et al., 2002).

The objective of the present study was to introduce a boiling stable protein (*bspA*) gene isolated from aspen into tomato by *Agrobacterium* mediated transformation and to screen transgenic plants for enhanced resistance to drought/osmotic stress. This novel 66 kDa boiling stable protein was highly expressed in response to gradual water stress, just an hour after initiation of drought treatment in aseptically reared shoots of *Populus tremula* (Pelah et al., 1995, 1997; Wang et al., 2002). It accumulated during progressive water stress but decreased on rehydration. The N-terminal amino acid sequence of BspA exhibited high homology to wheat germins GF-2.8 and GF-3.8 (Pelah et al., 1995). Although this protein does not share sequence similarities with known water stress and ABA responsive proteins, it does show some of their other features such as:

- accumulation in response to gradual water stress, ABA application and cold stress,
- heat stability,
- lack of cysteine,
- very low in tryptophan content as in cotton LEA proteins and
- highly hydrophilic constitution (Pelah et al., 1995; Wang et al., 2002). This is the first attempt, to the best of our knowledge, to transform tomato via *bspA* gene for drought tolerance.

Materials and methods

Bacterial strain and plasmid

The disarmed *Agrobacterium tumefaciens* strain EHA 105 harbouring a binary vector pBIG-HYG-*bspA* was generously provided by Professor Arie Altman (Hebrew University of Jerusalem, Rehovot, Israel). This plasmid contains the *bspA* gene, (later named as *sp1*; EMBL accession No. AJ276517) under the control of CaMV35S promoter (linked with TMV translational enhancer Ω downstream to the 35S promoter) and *nos* terminator, hygromycin phosphotransferase (*hpt*) driven by *nos* promoter and polyadenylation signal of *Agrobacterium* gene 7 as terminator (Figure 1). The pBIG-HYG-*bspA* was constructed as follows: The plasmid pJD330 harbours a *gusA* gene driven

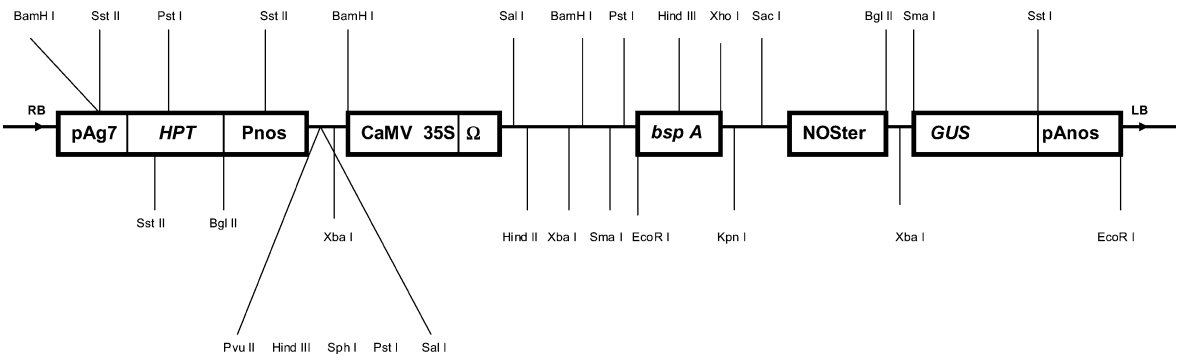

Figure 1. Map of the binary vector plasmid pBIG-HYG carrying the *bspA* cDNA. The vector contains *bspA* (boiling stable protein) gene under the control of CaMV35S promoter (linked with TMV translational enhancer Ω downstream to the 35S promoter) and *nos* terminator; *HPT* (hygromycin phosphotransferase gene) driven by *nos* promoter and polyadenylation signal of *Agrobacterium* gene 7 as terminator. The *GUS* gene present in the vector is promoterless. The figure is not drawn to scale.

by a CaMV35S promoter fused to the TMV translational enhancer Ω downstream to the 35S promoter and linked to a *nos* polyadenylation signal. The *bspA* gene was inserted into pJD330 at *Sal* I and *Sac* I, replacing the *GUS* gene. The above cassette containing 35S-*bspA*-NOSter was then cut out by *Xba* I and transferred to pBIG-HYG at *Xba* I, without taking the *GUS* out of

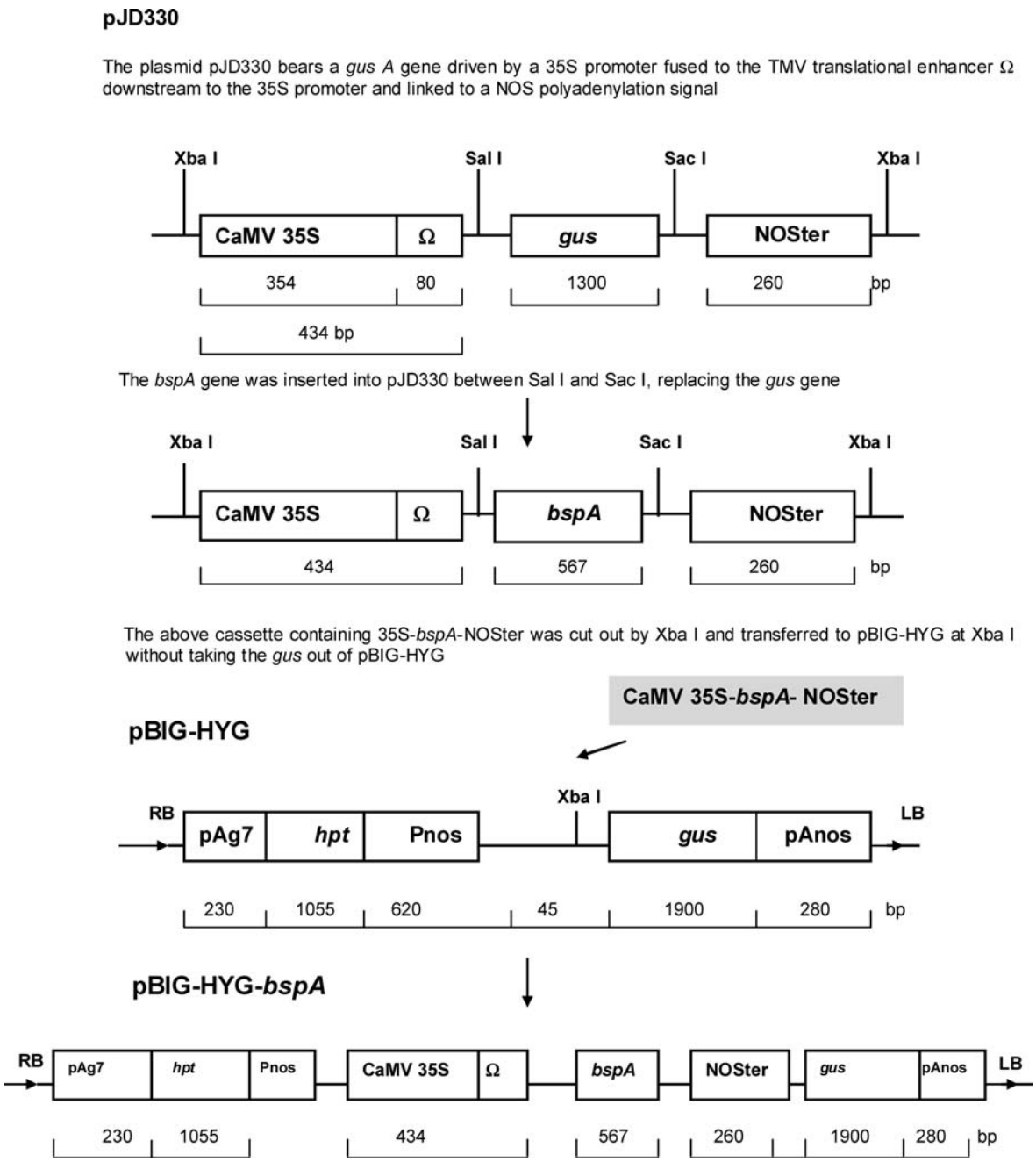

Figure 2. Flow chart showing stepwise construction of the binary vector pBIG-HYG-*bspA*. The plasmid pJD330 harbours a *GUS* gene driven by a CaMV35S promoter fused to the TMV translational enhancer Ω downstream to the 35S promoter and linked to a *NOS* terminator. The *bspA* gene was inserted into pJD330 between Sal I and Sac I, replacing the *GUS* gene. The above cassette containing 35S-*bspA*-NOSter was cut out by Xba I and transferred to pBIG-HYG at Xba I, without taking the *GUS* out of pBIG-HYG. The *GUS* gene present in the vector is promoterless. The figures are not drawn to scale.

pBIG-HYG (Roy et al. 2005). The *GUS* gene present in the vector is promoterless (Figure 2). *Agrobacterium* cultures carrying the *bspA* gene construct were grown overnight in LB broth containing 60 mg l^{-1} hygromycin and 30 mg l^{-1} rifampicin at 28 °C and maintained on a gyratory water bath shaker (New Brunswick Scientific Co., NJ, USA; Model G76D) providing 250 rpm.

Plant material

Lycopersicon esculentum Mill. cultivar 'Pusa Ruby' was used for transformation experiments. Seeds were procured from the National Seed Centre, Pusa Complex, New Delhi. They were surface sterilized by washing in water for 30 min and then treated with 1% Polysan (Polypharma Pvt. Ltd., Mumbai) for 10 min, 0.1% mercuric chloride (Qualigens, Mumbai) for 2 min and 95% ethanol for 30 s. Seeds were germinated on MS basal medium and leaf explants were excised from 6-week-old seedlings.

Co-cultivation of Agrobacterium tumefaciens *and tomato leaf explants*

Transformation was carried out according to the protocol of McCormik et al. (1986) with the modification that no feeder layer was used in the whole procedure. Approximately 2 cm^2 leaf explants were pre-conditioned for 2 days on regeneration medium, i.e. MS supplemented with 0.5 mg l^{-1} zeatin, 0.5 mg l^{-1} IAA, 30 g l^{-1} sucrose (Daurala, New Delhi) and 8 g l^{-1} agar (Qualigens, Mumbai) with abaxial side touching the medium. They were removed and gently immersed in cultures of *Agrobacterium* which were earlier grown overnight in LB broth and diluted in the ratio of 1:20 with MS liquid medium (O.D. approx. 0.1 at 660 nm). The leaf explants were dipped for 3 min in dark in *Agrobacterium* culture with constant shaking at 30 rpm until the leaf edges looked slightly wet. The leaf pieces were blotted dry between layers of sterile filter paper and returned to the same plates for a 2-day co-cultivation. Subsequently, the leaf explants were washed in MS liquid medium containing 500 mg l^{-1} cefotaxime, blotted dry and transferred to selection pressure medium (regeneration medium containing 40 mg l^{-1} hygromycin and 500 mg l^{-1} cefotaxime). Leaf explants along with developing calluses were sub-cultured after every 3 weeks on selection pressure medium till the shoots were formed. For inducing roots, shoots were separated from adhering callus and transferred to rooting medium (MS + 0.5 mg l^{-1} IAA) containing 40 mg l^{-1} hygromycin. Shoots that rooted in the presence of hygromycin were transferred to soil.

Polymerase chain reaction

Total genomic DNA was extracted from leaves of tomato plants using the SDS-extraction method (Dellapotra et al., 1983). The two primers for *bspA* gene were designed from both ends of the coding region. The forward primer sequence was 5′-AGA AAGGGAAGACATGGCAAC-3′ and the reverse primer sequence was 5′-CAGCATTTATTG AACATTACA-3′. The PCR reaction was carried out in a total volume of 25 µl comprising 100 ng of genomic DNA, 1x *Taq* buffer, 2 mM $MgCl_2$, 200 µM dNTPs mix, 0.5 unit *Taq* DNA polymerase and 90 pmol each of both the primers. The DNA was denatured at 94 °C for 2 min (hot start), followed by 36 amplification cycles (94, 62 and 72 °C each for 1 min). The amplified products were separated on 1.2% agarose gel.

Plasmid isolation and probe preparation

Plasmids carrying *bspA* gene were isolated from *E. coli* strain XL-1 Blue using the protocol of Birnboim and Doly (1979). Isolated plasmids were analyzed in 1% agarose gel stained with 0.5 µg ml^{-1} EtBr. The probe used for PCR-Southern hybridization and northern hybridization was the 496 bp PCR amplified fragment of plasmid DNA labelled with DIG-dUTP via random primer DNA labelling system. DIG labelling was carried out by the standard protocol of the manufacturer (Roche Molecular Biochemicals, Mannheim, Germany).

PCR-Southern hybridization

Following the capillary method (Southern, 1975), about 10 µg PCR amplified products of transgenic plants were transferred to nylon membrane and fixed for 7 min under UV cross-linker (120 mJ s^{-1}). Hybridization of the blots to digoxigenin (DIG)-dUTP labelled probe was carried out at 37 °C according to the manufacturer's instructions

(Roche Molecular Biochemicals, Mannheim, Germany). Using the DIG luminescent detection kit (Boehringer, Mannheim, Germany), it was detected and the hybridization signals were visualized by exposing Kodak X-ray film (X-OmatTM XK-5) at 37 °C for 30 min.

Northern hybridization

Total RNAs were extracted from young leaves by SV total RNA isolation system following manufacturer's instructions (Promega Corporation, Madison, USA). RNAs were denatured with formaldehyde as well as formamide and subjected to electrophoresis in 1.2% agarose gel. The separated RNAs were transferred to nylon membrane and hybridized with the probe following the procedure as described for PCR-Southern hybridization.

Drought tolerance tests of transgenic lines

All the drought tolerance tests were performed on leaves of 1-month-old plants of T_0 generation and each experiment was repeated thrice. The leaves of one plant were assayed thrice at different time intervals. Two transgenic lines of T_0 generation were evaluated in detail under water stress conditions. Leaves of non-transformed plants were used as control in all stress experiments.

Electrolyte leakage

The 24-h water stress was earlier determined as least duration for developing drought-induced proteins in tomato leaves (data not shown). Therefore, initially the non-transgenic and transgenic leaf discs (8 mm diameter) were water stressed separately by air-drying for 24 h. After 24 h water stress, they were washed for 5–10 min with distilled water to remove the surface adhering ions leached during the treatment and then immersed individually in fresh distilled water. The conductivity of the immersion solution (E_1) was measured after 15, 30, 45, 60, 120, 240 and 300 min by a digital conductivity meter (Electronic Instrumentation, Delhi, India). Control leaf discs of both, transgenic and non-transgenic plants were kept in water throughout the experiment. Control leaf discs were immersed in fresh distilled water before measuring electrical conductivity. To determine the total conductivity (E_2), the leaf discs with immersion solution (effusate) were autoclaved for 15 min at 121 °C and the conductivity of the effusate was measured after cooling it to room temperature. Relative electrical conductivity was measured by the formula $E_1/E_2 \times 100$.

PEG assay

To study whether the putative transgenic plants could resist reduced water potential, their leaf explants were cultured on regeneration medium supplemented with PEG 4000 in the range of 10–12% (severe water stress). 12% PEG 4000 was determined earlier to be the lethal dose for tomato leaf explants in the present study. Twenty-four explants were used per treatment and the experiment was repeated thrice. The leaf explants were initially weighed at the time of transfer. The fresh weight (FW) and dry weight (DW) of the leaf explants with developing calli and shoots were scored after 30 days of culture. DW of the tissue was obtained after oven drying at 80 °C for 24 h. The water content of the tissue was calculated as the (FW–DW)/DW ratio.

Proline estimation

Leaf explants with developing calli and shoots cultured on PEG supplemented medium for 30 days were selected for proline estimation. Proline was estimated according to the protocol of Bates et al. (1973).

Results

Lethal dose

The lethal dose of hygromycin for the control leaf explants was determined to check their intrinsic resistance. Regeneration was inversely proportional to the concentration of hygromycin. Regeneration was reduced and delayed on 10 mg l^{-1} but on 20 to 30 mg l^{-1} most of the explants started turning yellow and eventually brown without any regeneration. Callus developed in a few explants after a month of culture. No morphogenic response was elicited at 40 mg l^{-1}, and higher concentrations. On 40 mg l^{-1} they turned brown within one week, beyond which (50 and

60 mg l^{-1}) they showed bleaching. Thus, 40 mg l^{-1} hygromycin was selected as lethal dose for leaf explants.

Regeneration and selection of transgenic shoots

Among the 200 co-cultivated leaf discs, 52 began to differentiate green translucent callus at cut ends in 8–9 weeks on selection medium. Four weeks later, multiple shoots differentiated via calli. When the shoots elongated to 2–3 cm, they were excised and transplanted to rooting medium containing 40 mg l^{-1} hygromycin. Nearly 80% of the putative transgenic shoots survived and formed well-branched roots after 5–8 days of transfer. The plants were later transferred to soil where they flowered normally. The non-transformed leaf explants turned brown and did not show any morphogenic response even after 6 months of culture.

PCR analysis

To eliminate the potential of false positives arising from persistent *Agrobacterium*, the cultures were transferred to cefotaxime-free selection medium. No *Agrobacterium* appeared on this medium even after 30 days or longer. As expected, PCR analysis detected a 496 bp band in transgenic plants developed on root induction medium while none was detected in non-transformed plants (Figure 3).

PCR-Southern hybridization

Prior to attempting PCR-Southern hybridization, dot blot hybridization was carried out with DIG-dUTP labelled probe to test the integration of *bspA* gene in genomic DNA. DNA extracted from nine transgenic plants was dot blotted on positively charged nylon membrane (Boehringer, Mannheim, Germany) following the protocol described by Sambrook et al. (1989). Very strong signals were obtained in dots, which hybridized with transgenic plants while none in the control plant. Thus, dot blot analysis also confirmed the integration of *bspA* gene in transgenic plants (Figure 4a). PCR-Southern analysis with DIG-dUTP labelled probe showed excellent consistency with PCR results. PCR-Southern hybridization of amplified DNA products from transgenic plants with DIG-dUTP labelled probe confirmed the stable integration of *bspA* gene. PCR-Southern hybridization was performed on 15 PCR positive hygromycin resistant plants with the non-transformed plant as control. As expected, the blot containing PCR amplified products of transgenic plants showed a signal of 496 bp band in positive control as well as transgenic plants after overnight hybridization with DIG labelled probe while none was observed in the control plant (Figure 4b). The results of PCR, dot blot and PCR-Southern hybridization demonstrate that *bspA* gene has been integrated into the genome of the transgenic lines.

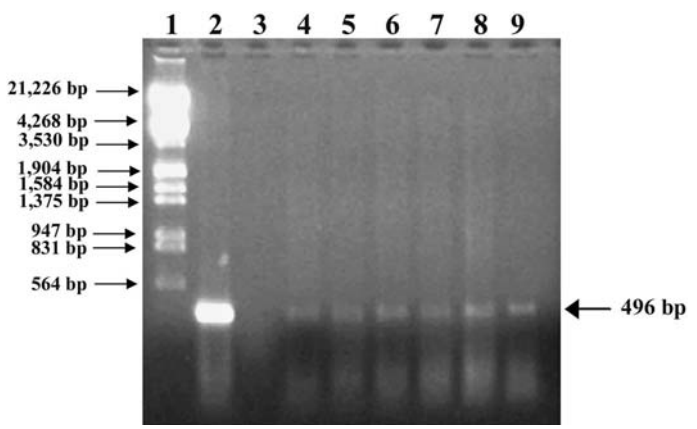

Figure 3. DNA separation in 1.2% agarose gel after PCR amplification of hygromycin resistant, *bspA* gene harbouring transgenic tomato plants. The 496 bp DNA fragment was amplified using gene specific primers. Lane 1: λ DNA/EcoR1+ Hind III marker, Lane 2: Positive control (*bspA* gene in bacteria), Lane 3: Untransformed plant, Lanes 4–9: 6 plants representing independent transformed lines showing 496 bp amplified DNA fragment.

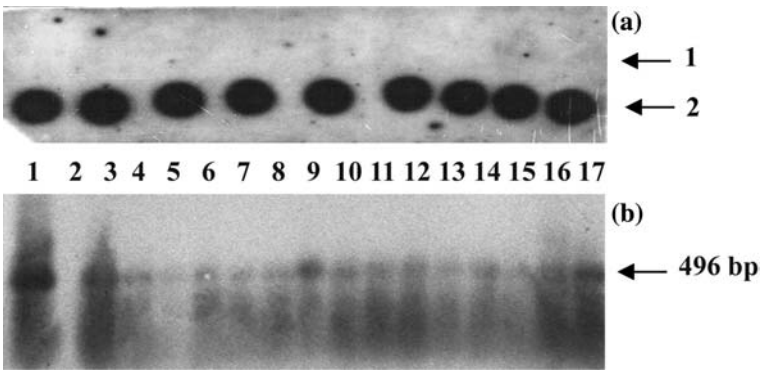

Figure 4 (*a*, *b*). Dot blot and Southern blot hybridizations in tomato. (*a*) Dot blot showing intense dots of transgenic plants (second row) while no signal is seen in control plants (first row) after overnight hybridization with DIG labelled probe. (*b*) Southern blot analysis of 15 transgenic lines of tomato transformed with pBIG-HYG-*bspA*. Lane 1: Positive control (*bspA* gene in bacteria), Lane 2: Untransformed plant, Lanes 3–17: 15 plants representing independent transformed lines showing the 496 bp segment.

Northern hybridization

Northern blot analysis showed presence of transcripts of *bspA* gene in transgenic plants but not in non-transgenic. However, the expression levels varied in different transgenic lines (Figure 5). Highest transcript level was observed in transgenic line 5–13 and lowest in line 2–17. Hence, these two lines were taken for drought tolerance tests.

Electrolyte leakage

The electrolyte leakage exhibited a characteristic pattern, reflecting conditions of cell membranes. It was observed that water stress resulted in increased ion leakage from leaf discs of non-transformed as well as transgenics compared with their respective controls (data not shown) but the rate and final value of electrolyte leakage from water stressed transgenic leaf discs was lower than those of the non-transgenics (Figure 6). The ion leakage among transgenics was variable, as represented by two lines, i.e. 2–17 and 5–13 (Figure 6). The latter line 5–13 showed less ion leakage compared to the former.

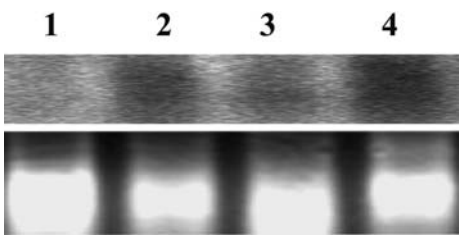

Figure 5. Northern blot analysis showing varying levels of transcripts of *bspA* gene in different transgenic lines. Lane 1: Untransformed plant, Lane 2: transgenic line 2–5, Lane 3: transgenic line 2–17, Lane 4: transgenic line 5–13. Lower panel shows ethidium bromide stained RNA gel.

PEG bioassay

The leaf explants of the transgenic lines and non-transgenic plant cultured on 10–12% PEG 4000 (severe water stress) containing culture media showed only slight differences in response. The biomass, in terms of both FW and DW, of the transgenic lines was higher than non-transformed (Figure 7a, b). As expected, the water content of the tissue decreased with PEG concentration in both, transgenic as well as non-transgenic plants. Although the difference was not significant but at each level, water content of transgenic tissues was higher than non-transgenics (Figure 8).

Proline estimation

The proline content was higher in transgenic plants under both unstressed and stressed conditions (Figure 9). The difference in the proline content was conspicuous at each level of PEG 4000.

Discussion

It is well known that stress induced proteins make plants more tolerant but direct evidence is still

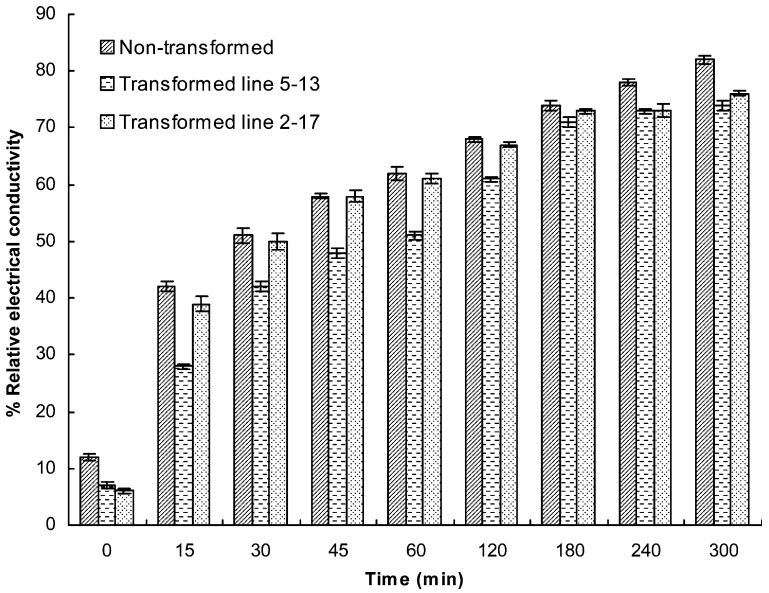

Figure 6. Relative electrolyte leakage percentages of water-stressed leaf discs of a non-transformed control and 2 transgenic tomato plants (line 2–17 and line 5–13). The results are expressed as averages of three experiments. Bars represent SD.

lacking since the functions of many stress responsive genes are unknown. Using transgenic approach, the functional role of these stress induced genes can be deciphered. Xu et al. (1996) reported that a ABA-inducible gene *HVA1* which encoded a group 3 LEA protein from barley seeds conferred drought tolerance to transgenic rice plants.

The objective of the present study was to investigate the role of drought induced *bspA* gene, isolated from *P. tremula*, in developing stress tolerance in tomato. The *bspA* gene encodes a hydrophilic boiling stable, ABA and stress-induced protein. An important strategy by which plants adapt or tolerate stress conditions is the expression of stress responsive proteins, which may function in plant tolerance (Dure, 1993). Proteins that accumulate during seed desiccation and in desiccated leaves include several belonging to families that are composed principally of a tandemly repeated 11-mer amino acid motif. An α-helical arrangement of the 11-mer repeating unit gives an amphiphilic helix whose hydrophobic stripe twists in a right-handed fashion around the helix. These proteins are highly hydrophilic and do not precipitate if boiled in aqueous solution (Piatkowski et al., 1990; Dure, 1993). This structure enables proteins to associate with membranal cracks caused by desiccation or cytoplasmic water loss during frost. They plug the cracks and prevent electrolyte leakage. They are involved in protection of enzymes against desiccation and structural proteins by protein–protein interactions. These interactions prevent the irreversible structural changes that occur in many proteins during water stress (Dure, 1993; Roberts et al., 1993). Such proteins function as water binding proteins, preventing complete water loss from membranes, and in sequestering excess ions, which accumulate in desiccated cells (Ingram and Bartels, 1996; Caruso et al., 2002).

Pelah et al. (1997) studied the differential expression pattern of BspA in poplar genotypes differing in water stress tolerance and reported a positive correlation between BspA accumulation and reduced ion leakage upon water stress. Wang et al., (1999) further studied the correlation between BspA expression and plant tolerance to water and salt stress in transgenic aspen plants overexpressing a full-length *bspA* cDNA fragment and reported a highly positive effect of BspA on stress tolerance.

In the present study, the stable integration of the *bspA* gene in tomato genome was confirmed by PCR and PCR-Southern hybidization. PCR was performed on six hygromycin resistant plants and it detected the expected 496 bp band of *bspA* gene

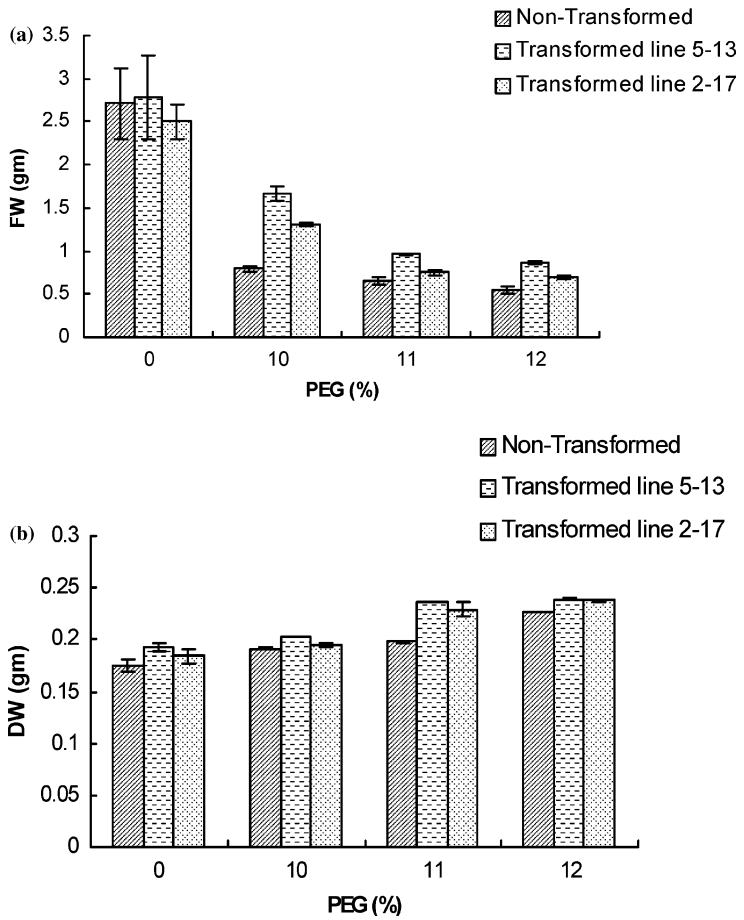

Figure 7 (*a*, *b*). Fresh (*a*) and dry (*b*) weights of non-transformed and the transgenic lines 5–13 and 2–17 cultured on 10–12% PEG 4000 supplemented regeneration medium for 30 days. The results are expressed as averages of three experiments. Bars represent SD.

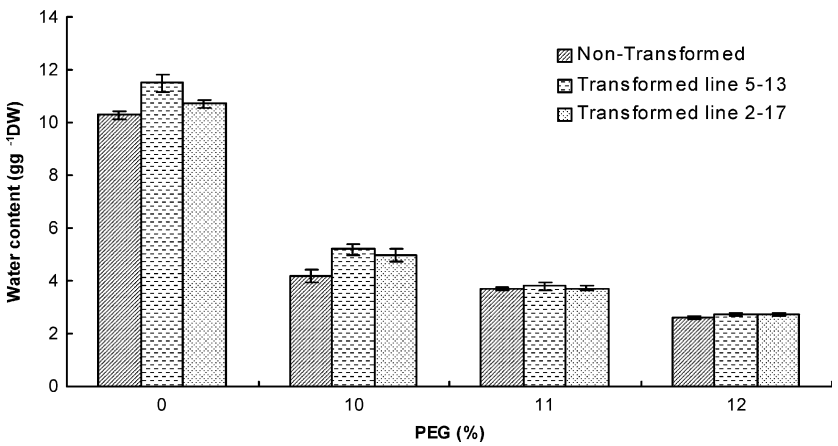

Figure 8. Water content of non-transformed and the transgenic lines 5–13 and 2–17 after 30 days of PEG 4000 treatment. The results are expressed as averages of three experiments. Bars represent SD.

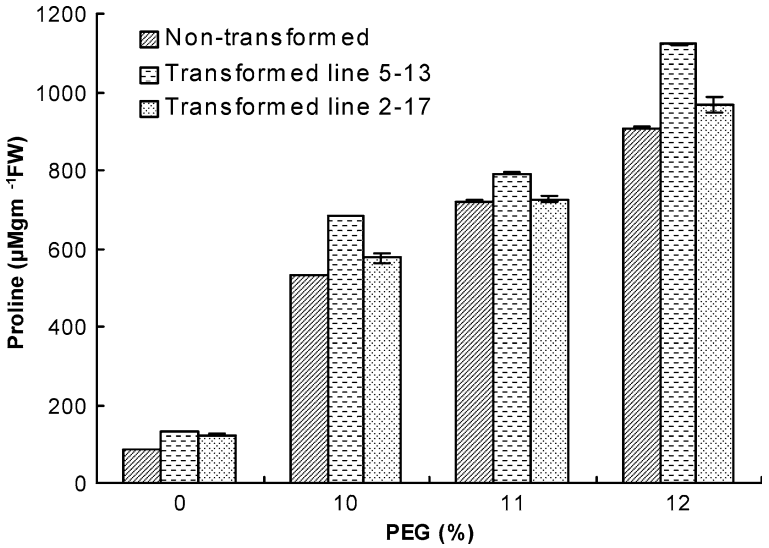

Figure 9. Proline content (µM gm^{-1} FW) of transgenic lines 5–13, 2–17 and non-transgenic plant after 30 days of culture on PEG 4000 supplemented regeneration medium. The results are expressed as averages of three experiments. Bars represent SD.

in transgenics, while it was not detected in control. PCR-Southern hybridization of 15 hygromycin resistant and PCR positive plants further confirmed integration of *bspA* gene in genome of transgenic lines investigated.

Several reports have shown that the transcript levels of transgenes increase by several folds during stress condition. In the present study, the *bspA* gene is driven by CaMV35S constitutive promoter (fused to the TMV translational enhancer Ω downstream to the 35S promoter), which can promote the expression of genes constitutively. In the northern blot analysis, the transcripts of *bspA* gene were detectable, no matter the drought stress was present or not but the expression levels were different among different transgenic lines. A direct correlation was observed between the transcript level and the observed stress tolerance of the transgenic lines. Maximum level of transcript was detected in the transgenic line 5–13 and the lowest level in 2–17; therefore, these 2 lines were taken for further studies on drought tolerance. As expected, it was observed that the transgenic line 5–13 showed more drought tolerance compared to the line 2–17 during these tests.

Adversities such as water and salt stresses damage the structure of cell membrane, making it leaky and resulting in outflow of ions so vital for cell functioning (Jia et al., 2002). Water stress-induced ion leakage can serve as a quantitative measure of stress-induced damage to cell membranes (Bramlage et al., 1978; Pelah et al., 1997), as cell membranes are one of the first targets of many plant stresses. The maintenance of integrity and stability of cell membrane under water stress conditions is a major component of drought tolerance in plants (Bajji et al., 2002). The results of electrolyte leakage analysis suggest that there was slight improvement in drought tolerance of transgenic plants compared to non-transgenics. Between the two transgenic lines, line 5–13 showed less leakage compared to line 2–17. The result showed consistency with the transcript levels detected in northern blot analysis as higher transcript level was recorded in line 5–13 and less in line 2–17. Low electrolyte leakage in transgenics compared to non-transgenics could be attributed to the expression of the boiling stable protein whose structure enables the protein to associate with membranal cracks caused by desiccation and prevent electrolyte leakage by plugging them as described above.

PEG test also showed slight improvement in regeneration from transgenics under severe osmotic stress compared to non-transgenics. PEG is a non-penetrating and non-ionic stress inducing compound. It is extremely hydrophilic organic polymer, which is able to remove much of the free

water within a solution. Therefore, it does not cause any ionic stress but only osmotic stress. PEG stressed transgenic lines (severe water stress by 10–12% PEG 4000) resulted in higher FW and DW compared to non-transgenics. Higher biomass of transgenics in terms of fresh and DW in PEG assay could also be attributed to the expression of boiling stable protein, which protected the enzymes against desiccation, and structural proteins by protein–protein interactions. This interaction possibly prevented the irreversible structural changes of enzymes responsible for carrying out various metabolic activities and thus this interaction prevented impaired cellular metabolism caused by water stress. Osmotic stress induced growth reduction has been reported to be more when considered on FW basis (Handa et al., 1983). Similar results were obtained in the present study. The reduction in FW of transgenic line was less compared to non-transgenic on PEG supplemented medium. There was not much difference between the DWs. A gradual increase of DW of tissues grown on PEG supplemented media followed by a steady growth indicated occurrence of a new homeostatic equilibrium which was probably compatible with the imposed stress.

Water content of the transgenic tissue was also recorded higher than the non-transgenic tissue under PEG stress. Although the difference was not significant, this indicated a positive effect of *bspA* gene in the transgenic line, which indicates *bspA* transgene enhanced the water uptake/retention capacity of plants under stress.

During different abiotic stresses, most of the plants accumulate high concentration of compatible solutes or osmolytes such as proline, fructan, mannitol, trehalose, glycinebetaine, etc. In the present investigation, proline content was estimated to study whether the expression of *bspA* gene was correlated to proline levels. The results indicated that genes for proline synthesis are upregulated in *bspA* transgenic tomato plants because proline content was higher in transgenic plants both under stressed and unstressed conditions.

All these tests indicated that plants transformed by *bspA* showed slightly enhanced resistance to drought tolerance. But the transgenic tomato plants did not become significantly tolerant to water stress because *bspA* is only one of the genes that are expressed in tomato and a combined activity may be required for drought tolerance. A possibility is that introduced *bspA* gene is necessary but not sufficient for osmoprotection and can only work in cooperation with other substances such as osmolytes. Only slight increase in tolerance of transgenics to water stress may also be attributed to low expression of the *bspA* gene (Reddy et al., 2003). The level of expression of transgene may be influenced by integration in different chromosomal positions, copy number, subsequent rearrangements, methylation of promoter or coding region and physiological or developmental stages of individual transgenic lines.

In the present investigation, only two plants of the T_0 generation were analyzed in detail but the results indicated that *bspA* is a promising candidate gene for improving drought tolerance. Further studies on a large number of plants of T_1 and T_2 generation are necessary to measure their response to water stress in field.

It is important to evaluate individual gene function by genetic engineering but also difficult to imagine that the expression of a single gene in transgenic plants could bring about a dramatic enhancement in stress tolerance, leading directly to new drought tolerant cultivar (Gisbert et al., 2000). Improving drought tolerance of plants by metabolic engineering is still a challenge because it is controlled by a network of genes (Shinozaki and Yamagauchi-Shinozaki, 2000; Altman, 2003). Tolerance to water stress could involve multigene families or a transcription factor regulating several genes as has been shown in *Arabidopsis* (Shinozaki and Yamagauchi-Shinozaki, 2000). Genetic stability of *bspA* transgenic tomato is under study and attempts are being made to bring about multigenic transformation to enhance drought tolerance of tomato plants.

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