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Heat stress protection in Aspen *sp1* transgenic *Arabidopsis thaliana*

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It is known that the stable protein 1 (SP1) detected in aspen plants remains soluble upon boiling and that *sp1* expression in transgenic aspen is resistant to salt stress. Presently, we analyzed the effect of expression of SP1 in *Arabidopsis thaliana* plants and their response to high temperature stress. After 45°C for 16 h, relative to wild type plants, *sp1* transgenic plants exhibited stronger growth and were better in several physiological properties including chlorophyll, chlorophyll fluorescence, water content, proline content, and malondialdehyde content. These preliminarily results suggest that the over-expression of SP1 may notably enhance heat-tolerant level of transgenic *A. thaliana* plants. [BMB reports 2008; 41(5): 382-387]

INTRODUCTION

Abiotic stresses such as drought, salinity, extreme temperature, chemical toxicity, and oxidative stress are serious threats to agriculture, and result in the deterioration of the environment. Abiotic stress reduces average yields for most major crop plants by more than 50% (1). High temperature stress (HTS) is one environmental factor that limits plant growth (2). The adverse effects of HTS on plant reproduction have implications on global crop production, in addition to contributing to the geographical distribution of natural plant species (3).

Populus is an important model organism in forest biology (4) that has become the study of much study. Stable protein 1 (SP1) was first isolated from aspen (*Populus tremula*) (5); this study reported that SP1 is a stress-related protein with no significant sequence homology to other stress-related proteins and may represent a new protein family. It is hydrophilic and remains soluble upon boiling like LEA-type proteins, and exhibits the oligomeric structure of small heat shock proteins (sHsps), representing a new class of plant proteins involved in the plant's response to abiotic stress. Biochemical analysis demonstrated that SP1 functions as a molecular chaperone in protecting and repairing different heat-labile enzymes (6). A thermostability study of SP1 using differential scanning calorimetry (DSC) and sequence and structural comparisons between SP1 and other stress-related proteins such as sHsp suggest that SP1 represents a new class of stress related proteins (7). Furthermore, *sp1*-transgenic aspen tolerates NaCl stress better than wild type plants (8).

In this report, we focused on the heat tolerance associated with over-expression of SP1 in transgenic *Arabidopsis thaliana*. We show direct evidence for the anti-high temperature stress role of SP1 in *A. thaliana*. We synthesized the recombinant gene, named *sp1l*, without changing its amino acid sequence through the PTDS (PCR-based two-step DNA synthesis) method (9). Then, we transformed the gene into *A. thaliana* through the floral dip method (10). The resulting transgenic plants that over-expressed *sp1l* exhibit higher survival rates after HTS.

RESULTS

Synthesis of the *sp1l* gene of *Populus tremula*

sp1 was synthesized by the PTDS method (9). Errors in the synthetic gene were corrected by the overlap extension polymerase chain reaction (OE-PCR) method (11, 12). We synthesized a recombinant gene, named *sp1l*, on the basis of the cDNA sequence of the wild type gene (GenBank accession no. AJ276517) from *Populus tremula*. Seven 60 nt oligonucleotides and one 70 nt oligonucleotide were used to synthesize the recombinant gene (Supplemental Table 1). The experimental details of the OE-PCR are outlined in Supplemental Fig. 1. Six inner oligonucletides (1.5 pmol) and two outer oligonucletides (30 pmol) were assembled to synthesized the *sp1l* fragment. BLAST search showed that the synthesized recombinant *sp1l* gene was 100% identical to the wild type *sp1* gene.

High temperature tolerance of transgenic plants

Through selection, we obtained transgenic plants that were identified by RT-PCR (Fig. 2A). We selected four transgenic

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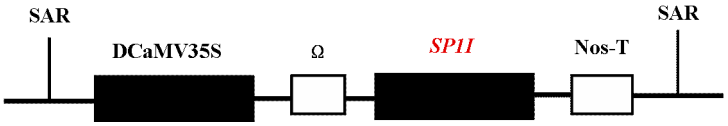

Fig. 1. Schematic diagram of the vector used in this study. The vector contains the double CaMV 35S (DCaMV35S) promoter and the tobacco mosaic virus (TMV) sequence fused to the sp1I gene. For steady transmission of the sp1I gene, two Scaffold attachment regions (SAR) were fused upstream of the DCaMV35S promoter and downstream of the Nos-Terminator (Nos-T).

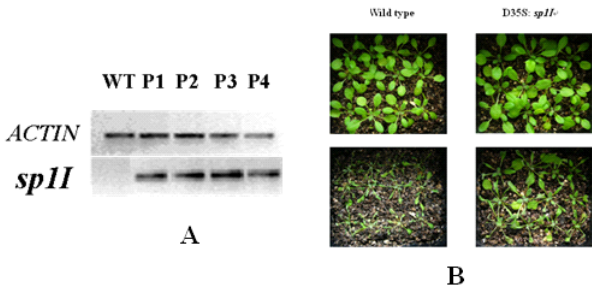

Fig. 2. Genomic integration and heat tolerance of transgenic plants. (A) Confirmation of *sp1I* genomic integration in transgenic lines by RT-PCR. Wild type (lane 1, WT) and transgenic lines (lanes 2-5) of *A. thaliana* were used as PCR templates. Specific primer pairs for PCR of *sp1I* were used. (B) Heat tolerance of the D35S:*sp1I* transgenic *A. thaliana*. Three-week-old wild type and transgenic plants grown in normal conditions and after 45°C ± 1°C for 16 h high temperature treatment were examined.

lines (P1-P4, corresponding to lanes 2-5, respectively, in Fig. 2A) that demonstrated relatively high expression of *sp1I*. The wild type and transgenic plants grown in pots were transferred to 45°C ± 1°C for 16 h and then returned to 22°C for 1 day; almost all of the wild type plants died, whereas 84% of the transgenic plants survived (Fig. 2B).

Chlorophyll content

To test if increased HTS may provide stress resistance, we examined the effects of high temperature on the total chlorophyll content in the leaves of seedlings. We selected three transgenic lines (P1, P2, and P3) and wild type plants for the experiment. The contents of total chlorophyll in wild type and transgenic plants were markedly decreased after stress. However, the decrease in the transgenic plants was significantly lower than that in wild type plants (Fig. 3A).

Determination of the chlorophyll fluorescence (Fv/Fm)

Chlorophyll fluorescence has been routinely used for many years to monitor the photosynthetic performance of plants non-invasively and to screen of plants for tolerance to environmental stresses (13). HTS damage on *sp1I*-expressing plants was evaluated both as photosystem II (PSII) stability and as injury to the whole plant. Fv/Fm was used to estimate the quantum yield of PSII photochemistry.

Wild type and transgenic plants were exposed to an excess of light energy at high temperature and Fv/Fm was measured at a different temperature. High temperature treatment caused a marked inhibition of PSII, as indicated by a decrease in the Fv/Fm value. However, the Fv/Fm value for wild type plants was dramatically lower than transgenic plants, not only in normal conditions but also after stress (Fig. 3B). This is consistent with the suggestion that plants over-expressing the *sp1I* gene were more tolerant to photo-inhibition than wild type plants.

Table 1. Differences in water content for wild type and transgenic plants

Line	Water content (% FW)	
	22°C	45°C
WT	93.1 ± 0.04	90.1 ± 0.04
P1	93.9 ± 0.10***	90.8 ± 0.05***
P2	93.8 ± 0.05	90.1 ± 0.04
P3	94.0 ± 0.04***	90.8 ± 0.03***

FW = fresh weight. Data are the mean ± SD of three replicates. Wild type and transgenic lines (P1 to P3) were grown in the same conditions. Asterisks indicate a significant difference ($P < 0.05$) compared to wild type plants in normal conditions and after high temperature treatment.

Water content

To detect the difference in growth of the wild type and transgenic plants, we quantified the water content of seedlings at different temperatures. The results are displayed in Table 1. HTS reduced plant water content significantly by an average of 3.24 % compared with plants at 22°C. Transgenic plants had increasingly higher water content than wild type plants, not only in normal conditions but also after stress.

Proline content

Proline appears to be the most widely distributed osmolyte accumulated under stress conditions (14). To verify that HTS modified the internal amino acid content, the proline contents of wild type and transgenic plants at different temperatures were measured (Fig. 3C). Proline contents were much lower in normal conditions, with 0.2072 μmol g^{-1} for wild type and 0.3226 μmol g^{-1} at maximum for the transgenic plants. However, after stress the maximal proline content was much higher, at 3.1239 μmol g^{-1} for the wild type and 5.2042 μmol

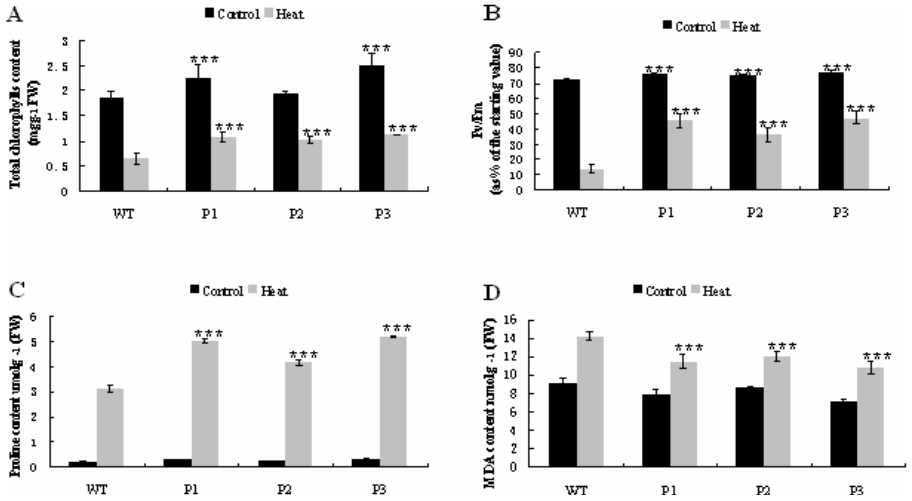

Fig. 3. Effects of high temperature on the physiological properties in leaves of wild type and transgenic plants. Data are the mean ± *SD* of three replicates. The wild type plants and transgenic lines (P1 to P3) were grown in the same conditions. Asterisks indicate a significant difference ($P < 0.05$) compared to wild type plants in normal conditions and after high temperature treatment.

g^{-1} for the transgenic plants. The increase in the transgenic plants was significantly higher than that in wild type plants.

Lipid peroxidation

The level of lipid peroxidation for wild type and transgenic plants was measured at different temperatures on the basis of the accumulation of malondialdehyde (MDA), a major product of lipid peroxidation (15). As shown in Fig. 3D, significant differences in the levels of MDA were detected among wild type and transgenic plants. It was lower in normal conditions than after stress; however, the MDA concentration of wild type was somewhat higher than transgenic plants. After stress, a significant reduction in the accumulation of lipid peroxidation-derived reactive aldehydes was observed in transgenic plants as compared to wild type plants.

DISCUSSION

In this paper, we studied *sp1* function upon transfer of this gene into *A. thaliana*. We found that high temperature treatment caused significant declines in chlorophyll content, water content, and Fv/Fm in both wild type and transgenic lines (Table 1, Figs. 3A and 3B). Moreover, chlorophyll content, water content, and Fv/Fm were lower in wild type compared to transgenic lines both in normal and high temperature conditions. The detrimental effects were more pronounced for wild type than transgenic lines.

Chlorophyll content in live plants is an important factor in determining growth capacity. Chlorophyll fluorescence analysis has become one of the most powerful and widely used techniques available to plant physiologists and ecophysiologists (16). Zoran *et al*. studied the relationship between the heat stability of thylakoids and loss of chlorophyll in winter wheat (*Triticum aestivum L.*) under heat stress (17). Our findings are consistent with their results that heat stress cause a decline in chlorophyll content; moreover, heat-induced damage to thylakoids and chlorophyll loss are closely associated. Although the present measurements of water content were not significant between transgenic and wild type plants, the former did exhibit higher water content than wild type plants. Moreover, another study found 0.25 mmol salicylic acid (SA) to be most effective in enhancing heat tolerance in Kentucky bluegrass, which was manifest by improved re-growth potential following heat stress of 72 h and maintenance of leaf water content at 77% during the 12 h stress period similar to that under normal temperature conditions (18). Heat stress was associated with a 3.6% higher water content in Kentucky bluegrass plants as compared with untreated plants (18). Our result is consistent with this water content change, and strengthens the view that the water content in plants may be tied to heat tolerance.

Proline accumulation is another important factor in determining heat tolerance that can have a major role in osmotic adjustment (19) among several other potential protective roles. Plant survival in most environments requires their ability to withstand extremes of osmotic stress caused by drought, salinity, and temperature. They have evolved two major mechanisms for accomplishing this: water stress avoidance and tolerance (20-22). In the present study, proline content in transgenic plants was higher than in wild type plants (Fig. 3C) and transgenic plants also showed normal growth after stress. In transgenic tobacco plants that over-express the mothbean (*Vigna aconitifolia*) *p5cs* gene, 10-18 times more proline is produced than in wild type plants (23). Our proline content finding is consistent with these results. Therefore, proline accumulation is important for heat tolerance in transgenic plants. Although the physiological function of SP1 at a molecular level has not yet been identified, here we show that *A. thaliana* plants over-expressing *sp1l* have higher survival rates after HTS. The stress tolerance mechanisms could be due to active accumulation of proline. Further characterization is still need-

ed to determine the specific molecular and physiological functions of SP1 in stress tolerance.

The accumulation of MDA often is used as an indicator of lipid peroxidation (24). HTS increased MDA content both in wild type and transgenic lines (Fig. 3D), similar to what has been found in other species (25). The results indicate that membrane lipid peroxidation occur from the malfunction of the scavenging system, which can prelude damage to main cellular components (26). Less MDA accumulated in transgenic lines than in wild type plants, suggesting that less lipid peroxidation developed because the *sp1l* gene was over-expressed in transgenic plants at high temperature.

In this paper, transgenic plants over expressed *sp1* gene were identified by RT-PCR, (Fig. 2A); the specific band was detected in transgenic plants but not found in wild type plant. Moreover, 84% of plants over-expressing *sp1l* could survive after HTS, whereas almost all of the wild type plants died (Fig. 2B). We also preliminarily examined tolerance to chilling, dehydration and salt stresses in the transgenic plants, but did not observe significant enhancement of such tolerances (data not shown). NaCl resistance in *sp1*-transgenic aspen (8) suggests that different species difference induce different results.

MATERIALS AND METHODS

Design and chemical synthesis of *sp1l*

Polymerase chain reaction (PCR) was carried out with 1.5 pmol of each of six inner oligonucleotides and 30 pmol of each of two outer oligonucleotides for 25 cycles with 2.5U *pyrobest* polymerase (TaKaRa, Dalian, China). The conditions of this PCR-mediated assembly were 30 s at 94°C, 30 s at 45°C, and 30 s at 72°C for each cycle followed by an additional 10 min at 72°C to ensure complete extension for all PCR reactions.

Transgenic plants over-expressing synthesized *sp1l* cDNA

The synthesized *sp1l* gene was cloned into the binary vector pYG8401, which was constructed on the basis of the vector pCAMBIA-1301, with the hygromycin gene as the genetic selection marker. The *sp1l* gene cassette containing the double CaMV 35S promoter and Nos-Terminator in its downstream sequence was flanked by two tobacco scaffold attachment regions (Fig. 1). The constructs were introduced into *Agrobacterium tumefaciens* GV3101 by electroporation. *A. thaliana* was transformed by the floral dip method (10).

Plant materials

Seeds of *A. thaliana* were surface sterilized with bleaching powder (5%, w/v) for 20 min, washed with sterile water three times, and placed in Petri dishes that contained MS (27) medium with 0.8% agar. The incubation and growth conditions of *A. thaliana* were the same as described previously (10).

Reverse transcription-PCR

Total RNA was digested with DNase I (Promega, Madison, WI, USA) to remove genomic DNA. The first strand of cDNA was synthesized using 5 μg of total RNA as template with the Reverse Transcription System (Promega) in a 20 μl reaction volume. In order to improve the reliability of RT-PCR, the *A. thaliana* actin gene (AtAc2, accession number NM112764) synthesized by two primers (AtAc2Z1: 5′-GCA CCC TGT TCT TCT TAC CGA G-3′; AtAc2F1: 5′- AGT AAG GTC ACG TCC AGC AAG G-3′) was used as an internal standard gene. PCR was carried out in 27 cycles of 40 s at 94°C, 30 s at 58°C, and 20 s at 72°C, plus a final extension at 72°C for 5 min. A 201-bp fragment of *sp1l* was amplified using two specific primers (*sp1l*Z1: 5′- AAT GAC TAT ACC AAT CTG CTC GA-3′; *sp1l*F1: 5′- CAA AGT AGG CAA AAA CCC TTC TG -3′) according to the sequence of the *sp1l* gene, and PCR reaction conditions were the same as AtAc2. The PCR products were separated on 2% agarose gel and quantified using a Model Gel Doc 1000 (Bio-Rad, Hercules, CA, USA). DNA intensity ratio of *sp1l* to AtAc2 was analyzed with ShineTech Gel Analyser (Shanghai Shine Science of Technology, Shanghai, China) to evaluate the *sp1l* expression pattern. The experiments were repeated three times with the same results; data from a representative experiment is presented.

Determination of chlorophyll content

Chlorophyll was extracted from individual leaves with 95% ethanol and its content determined as described previously (28).

Fv/Fm measurements

Photochemical efficiency of photosystem II (PSII) determined by Fv/Fm and chlorophyll fluorescence emission from the upper surface of the leaves was measured with a Model PAM-2100 plant efficiency analyzer (Heinz Walz GmbH, Effreltrich, Germany) as previously described (29). Before measurements, leaves were dark-adapted for 30 min at room temperature.

Water content

After the fresh weight (FW) was measured, whole plants were dried at 110°C for 1 h to kill tissue quickly and then dried at 70°C for 24 h in a forced-air oven. Dried plants were weighted after being cooled to room temperature in a desiccator for 0.5 h.

Determination of proline content

Proline content was measured as previously described (30).

Detection of lipid peroxidation

The extent of lipid peroxidation in leaves was estimated by measuring the amount of MDA, a decomposition product of the oxidation of polyunsaturated fatty acids, as described previously (31, 32).

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