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# A Possible Role of Trehalose as a Regulatory Molecule in Plant Drought Resistance

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**ABSTRACT : In many organisms, trehalose has been known as an energy source and a protectant against various environmental stresses such as desiccation, freezing, heat and osmotic pressure. Previously, we have isolated and characterized the genes encoding trehalose-6-phosphate synthase (ZrTPS1) and trehalose-6-phosphate phosphatase (ZrTPS2) from one of the most osmotolerant yeasts, *Zygosaccharomyces rouxii*. We have also generated transgenic plants by co-introduction of *ZrTPS1* and *ZrTPS2* into potato plant (ZrTPS2-2A-ZrTPS1 plant) in an attempt to metabolically engineer trehalose in the transgenic plant using the foot-and-mouth disease virus (FMDV) 2A system and to generate drought resistant crop plants. In this research, we assayed previously generated the ZrTPS2-2A-ZrTPS1 plant biofunctionally by drought treatment, and measured the amount of trehalose in the ZrTPS2-2A-ZrTPS1 transgenic plants. The ZrTPS2-2A-ZrTPS1 transgenic plant showed strong drought resistance in spite of little or no accumulation of trehalose in the transgenic plant compared with control plant.**

**Key words:** trehalose, *Zygosaccharomyces rouxii*, ZrTPS1, ZrTPS2.

## INTRODUCTION

Trehalose is non-reducing disaccharide consisting of two glucose molecules. It is widely distributed among many organisms including bacteria, algae, fungi, plants, insects and invertebrate animals, especially among anhydrobionts[1,2]. Trehalose was known to have dual functions in yeast, *Saccharomyces cerevisiae*: as a storage carbohydrate and as a stress protection metabolite. Its role in stress protection is considered probably by preventing denature of enzyme and protein of cell membrane upon dehydration[3-5], freezing injuries[6,7], heat stress[8,9] and hyper-osmotic stress[10].

In yeast, trehalose is known to be synthesised in a two-step process. First, trehalose-6-phosphate synthase (TPS1) synthesizes trehalose-6-phosphate using UDP-glucose (uridine-diphosphate glucose) and glucose-6-phosphate. Second, trehalose-6-phosphate phosphatase (TPS2) converts trehalose-6-phosphate into trehalose by dephosphorylation[11].

Previously, we have cloned and characterized the genes encoding trehalose-6-phosphate synthase (ZrTPS1) and trehalose-6-phosphate phosphatase (ZrTPS2) from *Zygosaccharomyces rouxii*, one of the most osmotolerant yeasts[12]. We showed that ZrTPS1 and ZrTPS2 functionally complemented the *S. cerevisiae tps1* deletion mutant and the *tps2* disruption mutant, respectively[12].

In addition, we have also generated transgenic plants by co-introduction of *ZrTPS1* and *ZrTPS2* into potato plant (ZrTPS2-2A-ZrTPS1 plant) in an attempt to metabolically engineer trehalose in transgenic plant using foot-and-mouth disease virus (FMDV) 2A system and to generate drought resistant crop plants.

In this research, in order to study role of trehalose in drought tolerance of plants, we assayed previously generated the ZrTPS2-2A-ZrTPS1 transgenic plant biofunctionally by drought treatment, and measured the amount of trehalose in the ZrTPS2-2A-ZrTPS1 transgenic plants.

## MATERIALS AND METHODS

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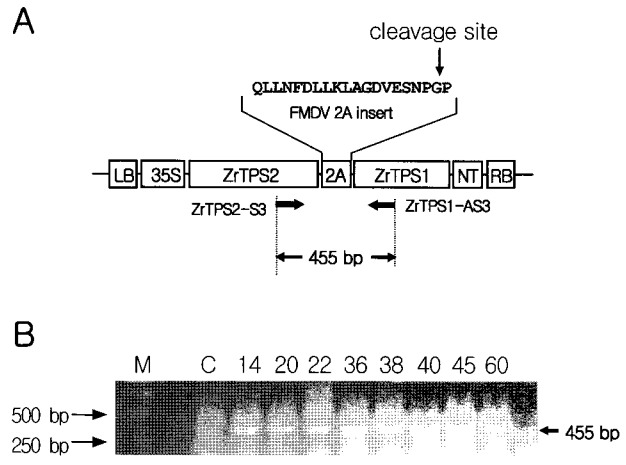


**Fig. 1. Binary vector construct and the expression of the recombinant *ZrTPS2-2A-ZrTPS1* gene.** A. Binary vector (pBI121 /ZrTPS2-2A-ZrTPS1) construction of the introduction of the *ZrTPS1* and *ZrTPS2* genes into potato plants. 35S, CaMV 35S promoter; NT, nopaline synthase terminator; 2A, FMDV 2A sequence. B. RT-PCR using ZrTPS2-S3/ZrTPS1-AS3 primers sets with RNA from the transgenic plants as template provided 455 bp PCR product. Lane M: 1 kb ladder. C: the control plant transformed with empty vector (pBI121). The numbers are the ZrTPS2-2A-ZrTPS1 transgenic lines.

### Plant materials

The transgenic potato plants (*Solanum tuberosum*, cv. Sumi) that was transformed with pBI121/ZrTPS2-2A-ZrTPS1 previously were used in this study (Fig. 1A). The transgenic potato plants were grown either in a controlled growth chamber or temperature controlled green house of 23± 1℃.

### Reverse-transcription polymerase chain reaction (RT-PCR)

In order to test the expression of ZrTPS2-2A-ZrTPS1 transcript in a single open reading frame in transgenic potato plants, total RNA was extracted using Tri Reagent (MRC, USA). RT-PCR was performed using RobusT I RT-PCR kit (Finnzymes, Finland). RT reaction was performed for 1 hr at 42℃ with 2 ㎍ of total RNA using ZrTPS1-AS3 primer (5'-CAAGACTTGGCCAACCATACCACTGG-3'). Immediately PCR reaction was performed using the following cycling parameters; 1 cycle of 2 min at 95℃, 35 cycles of 1 min at 95℃, 1 min at 55℃, 2 min at 72℃ and final extension of 10 min at 72℃. The primer sets used for PCR was primers ZrTPS2-S3 (5'-GTACTTGACACCTTGGGTCTA-CTTG-3') and ZrTPS1-AS3 combination. Sense primer was designed at +2500 bp downstream in *ZrTPS2* and antisense primers at +200 bp downstream in *ZrTPS1*. Thus, the resulting PCR products of 455 bp in length could be generated if the introduced recombinant ZrTPS2-2A-ZrTPS1 DNA transcribed as a single transcript (Fig. 1B).

### Drought treatment of the transgenic plants

To test the tolerance of the ZrTPS2-2A-ZrTPS1 transgenic potato plants to drought stress, the transgenic and the control potato plants growing in the soil mixture were withheld irrigation for 15 days.

### Trehalose content

Trehalose was measured in the control and the ZrTPS2 -2A-ZrTPS1 transgenic plants by high pressure liquid chromatography. Leaves of potato plants were harvested and frozen dried in 15 mL conical tubes. One gram of powdered samples were than resuspended in 10 mL of 85% ethanol by vortexing and incubated overnight. After centrifugation for 10 min at 3,000 ×g, the supernatant was concentrated to 1.5 mL volume followed by addition of 1.5 mL of water and celite. This was then centrifuged at 12,000 ×g. The supernatant was retrieved and kept at -20℃ until used. Samples (10 μL) were analyzed with 75% acetonitrile at 0.8 mL/min using HP HPLC1100 system with carbohydrate column (4.6×250 mm, Waters Co.) and guard column (Waters Co.).

## RESULTS

### Expression of the recombinant *ZrTPS2-2A-ZrTPS1* DNA

Expression of the recombinant *ZrTPS2-2A-ZrTPS1* gene in transgenic potato plants was detected by RT-PCR. Because the primer set used for this was designed one primer (ZrTPS2-S3) at *ZrTPS2* and the other primer (ZrTPS1-AS3) at *ZrTPS1*, the generation of RT-PCR products means the expression of recombinant *ZrTPS2-2A-ZrTPS1* gene as one single reading frame (Fig. 1B). No transgene expression was observed in the empty vector control plants (Fig. 1B). Among 70 plants which were confirmed transgene insertion into plant genome, six plants showed no transgene expression at all (data not shown). Regardless of the transgene expression level, the transgenic potato plants did not show any morphological aberration.

### Drought treatment of the ZrTPS2-2A-ZrTPS1 transgenic plants

The biofunctional analysis of the ZrTPS2-2A-ZrTPS1 transgenic potato plants was carried out by drought treatment. To test enhanced drought tolerance of the ZrTPS2 -2A-ZrTPS1 transgenic potato plants, the empty vector con-

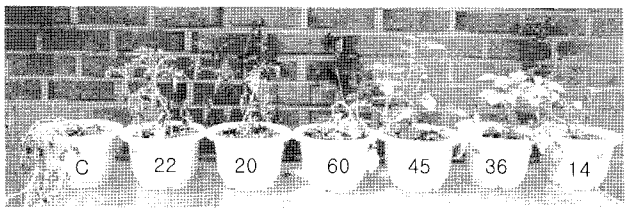


**Fig. 2. Drought tolerance of ZrTPS2-2A-ZrTPS1 transgenic plants.** One month old plants were not watered for 2 weeks. C is the empty vector transgenic control plant, and the numbers are ZrTPS2-2A-ZrTPS1 transgenic lines.

trol and the ZrTPS2-2A-ZrTPS1 transgenic plants grown in the soil mixture were not watered for 15 days. There was a significant difference in drought resistance between transgenic and nontransgenic potato plants. The ZrTPS2-2A-ZrTPS1 transgenic potato plants showed nonwithering, while the empty vector control potato plants withered completely (Fig. 2). The transgenic lines showed normal growth phenotypes both in the soil mixture and in culture tubes. The transgenic lines 14, 20, 22, 36, 38, 40, 45 and 60 showed the strong resistance to drought. Among these, line 36, 38 and 45 showed the most resistance to drought. These lines remained unwithered following 20 days of withholding irrigation, at which time the water capacity of the soil was 3.5% (v/v).

### Measurement of trehalose

Increased drought resistance of the ZrTPS2-2A-ZrTPS1 transgenic potato plants compared with control plant drove us to measure the amount of trehalose in the transgenic plant that might be produced by introduced ZrTPS1 and ZrTPS2 genes. As shown in Fig. 3, the empty vector control plant contains 4.8 mg/g DW of trehalose. The ZrTPS2-2A-ZrTPS1 transgenic lines accumulated trehalose ranging from 4.0 to 6.1 mg/g DW. This indicates that the transgenic lines did not accumulate meaningful amount of trehalose compared to control plant. This is somewhat surprising result in that the transgenic plant showed increased drought resistance in spite of little or no trehalose accumulation.

## DISCUSSION

As one approach to use trehalose metabolism to generate abiotic stress resistant plants, we have isolated and characterized *ZrTPS1* and *ZrTPS2* genes from *Z. rouxii*[12]. We have also generated transgenic plants by co-introduction both of *ZrTPS1* and *ZrTPS2* into potato plant (ZrTPS2-2A-ZrTPS1 plant) in an attempt to metabolically engineer trehalose in transgenic plant using foot-and-mouth disease virus

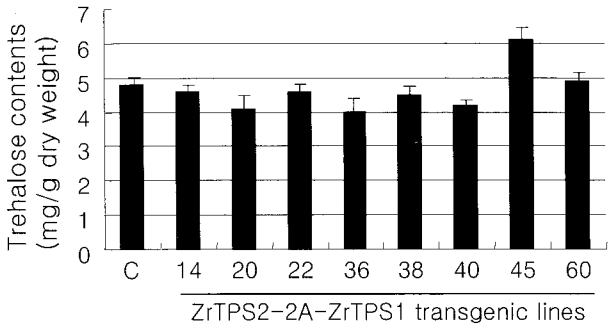


**Fig. 3. Trehalose contents in the ZrTPS2-2A-ZrTPS1 transgenic plants.** The trehalose contents were measured using HPLC from the leaves and stems of the control and the transgenic plants. C is empty vector control plant and the numbers on the Y-axis are the ZrTPS2-2A-ZrTPS1 transgenic lines. Each value is the mean±SD (n=3 individual plants).

(FMDV) 2A system and to generate drought resistant crop plants. Previously, we have introduced the *TPS1* gene of *S. cerevisiae* into potato plants to engineer drought tolerant plants[14]. It appeared that the resulting TPS1 transgenic plants were highly resistant to drought stress. However, when trehalose contents was measured, there was little or no trehalose accumulation observed. Also, some of the transgenic lines showed phenotype alteration. The reason for this might be the absence of its own phosphatase (TPS2) or inefficiency or not enough endogenous phosphatase to change trehalose-6-phosphate into trehalose. Thus, it might be important that both the TPS1 and TPS2 genes were introduced at simultaneously into plants and maintained same expression level in order to increase the efficiency of drought tolerance of transgenic potato plants.

In this study we measured trehalose content ZrTPS2-2A-ZrTPS1 transgenic plant. All the transgenic plant did not accumulate significant amount of trehalose compared to control plants (Fig. 3). Because we did not perform enzyme assay we could not sure whether the transgenic plant had proper system to produce trehalose using introduced *ZrTPS1* and *ZrTPS2* gene. However, when we performed drought test, the transgenic plants showed strong resistance to drought stress compared to the empty vector transgenic control plants (Fig. 2).

In this research, we tried to use FMDV 2A system to co-introduce both the *ZrTPS1* and *ZrTPS2* genes into potato plant using. FMDV 2A consisting of 16~20 amino acids is self-cleavage polypeptide. Thus, it could be expected that the introduced recombinant *ZrTPS2-2A-ZrTPS1* gene could be transcribed in a single reading frame. Then, the *ZrTPS2-2A-ZrTPS1* transcript could be translated into ZrTPS2-2A-ZrTPS1 polypeptide. Because FMDV 2A polypeptide has

capability to mediate cleavage at its own C-terminus by an enzyme independent reaction, the resulting ZrTPS2-2A-ZrTPS1 polypeptide was expected to be cleaved by 2A polypeptide generating two functional proteins, ZrTPS1 and ZrTPS2. This system has great advantage of same expression level of two co-introduced genes. Because we designed one primer from ZrTPS2 and the other from ZrTPS1 for RT-PCR, the resulting RT-PCR product could confirm generation of recombinant *ZrTPS2-2A-ZrTPS1* mRNA as a single transcript (Fig 1B). Although the ZrTPS2-2A-ZrTPS1 transgenic plant showed increased drought tolerance compared to empty vector transgenic control plants, we could not confirm the generation of two independently functional ZrTPS1 and ZrTPS2 protein. For detection of the functional ZrTPS1 and ZrTPS2 production in the transgenic plant, enz- yme assay and/or Western blot analysis should be perfor- med. Currently, we are in the middle of generating ZrTPS1 and ZrTPS2 antibodies for protein analysis.

In addition that *ZrTPS2-2A-ZrTPS1* transgenic lines showed increased drought tolerance, ZrTPS2-2A-ZrTPS1 transgenic potato plants showed nor morphological alteration at both in the test tube and in the soil. Our previous results showed that TPS synthesizing transgenic plant showed great drought tolerance without accumulating trehalose[13]. Yeast TPS1 transgenic tobacco plant driven by constitutive CaMV 35S promoter appeared to accumulate very low level of trehalose, up to 170 ppm of fresh weight, and it showed increased drought resistance[14]. Tobacco plants expressing both the *E. coli otsA* and the *otsB* gene simultaneously showed also very low trehalose accumulation (8~9 ppm of fresh weight) and showed significantly increased drought resistance[15]. It is well known that trehalose functions as an effector molecule for various abiotic stress resistance. Thus, from our and other's results[13,16], we propose that trehalose may function not only as an effector molecule but also as a regulatory molecule for plant drought resistance. Intensive research should be performed to elucidate this possibility.

## ACKNOWLEDGEMENT

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