BMB
Reports

BMB Rep. 2014; 47(1): 27-32
www.bmbreports.org

Over-expression of *BvMTSH*, a fusion gene for maltooligosyltrehalose synthase and maltooligosyltrehalose trehalohydrolase, enhances drought tolerance in transgenic rice

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Plant abiotic stress tolerance has been modulated by engineering the trehalose synthesis pathway. However, many stress-tolerant plants that have been genetically engineered for the trehalose synthesis pathway also show abnormal development. The metabolic intermediate trehalose 6-phosphate has the potential to cause aberrations in growth. To avoid growth inhibition by trehalose 6-phosphate, we used a gene that encodes a bifunctional in-frame fusion (*Bv*MTSH) of maltooligosyltrehalose synthase (*Bv*MTS) and maltooligosyltrehalose trehalohydrolase (*Bv*MTH) from the nonpathogenic bacterium *Brevibacterium helvolum*. *Bv*MTS converts maltooligosaccharides into maltooligosyltrehalose and *Bv*MTH releases trehalose. Transgenic rice plants that over-express *BvMTSH* under the control of the constitutive rice *cytochrome c* promoter (101MTSH) or the ABA-inducible *Ai* promoter (105MTSH) show enhanced drought tolerance without growth inhibition. Moreover, 101MTSH and 105MTSH showed an ABA-hyposensitive phenotype in the roots. Our results suggest that over-expression of *BvMTSH* enhances drought-stress tolerance without any abnormal growth and showes ABA hyposensitive phenotype in the roots. [BMB Reports 2014; 47(1): 27-32]

INTRODUCTION

Trehalose (α-D-glucopyranosyl-[1,1]-α-D-glucopyranose) is a nonreducing diglucoside found in various organisms, including bacteria, algae, fungi, yeasts, insects, and some plants (1). Trehalose acts as a carbohydrate reserve and as a protector against various stresses in many organisms (2-4). A role for trehalose in drought-stress tolerance has been demonstrated in the resurrection plant *Selaginella lepidophylla* and desiccation tolerant angiosperm *Myrothamnus flabellifolius* (5, 6). The genes for trehalose biosynthesis were discovered in plants, and studies indicate that trehalose synthesis plays a role in stress tolerance in plants (6). During osmotic stress, trehalose, or similar carbohydrates, appears to be more important than proline (7). Until recently, many transgenic plants have been designed to have increased drought tolerance using trehalose biosynthesis genes because of the involvement of trehalose in abiotic stress tolerance (4).

Trehalose is biosynthesized through two enzymatic processes in various organisms, including higher plants. Trehalose 6-phosphate (T6P) synthase (TPS) converts UDP-glucose and glucose 6-phosphate to T6P, which is further dephosphorylated to trehalose by T6P phosphatase (TPP) (5). Plant abiotic stress tolerance has been modulated by engineering the trehalose synthesis pathway. However, many stress-tolerant plants that have been genetically engineered to contain the trehalose synthesis pathway also show abnormal plant development, such as dwarfism and an altered leaf phenotype (8-12). Although the reason is not clear, the metabolic intermediate T6P has the potential to cause growth abnormalities (13). Therefore, it has been postulated that producing trehalose without the T6P intermediate in plants could result in an increase in stress tolerance without growth aberrations.

In some bacteria, the biosynthesis of trehalose is mediated by maltooligosyltrehalose synthase (MTS) and maltooligosyltrehalose trehalohydrolase (MTH). In this pathway, T6P is not generated as an intermediate. One nonpathogenic bacterium, *Brevibacterium helvolum*, is known to contain these two enzymes (14). In this study, we generated transgenic rice plants that over-expressed the bifunctional in-frame fusion gene (*BvMTSH*) of *BvMTS* and *BvMTH*. Our results suggest that

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http://dx.doi.org/10.5483/BMBRep.2014.47.1.064

Received 19 March 2013, Revised 5 April 2013, Accepted 16 May 2013

Keywords: *BvMTSH*, Drought, Maltooligosyltrehalose, Maltooligosyltrehalose synthase, Trehalose

ISSN: 1976-670X (electronic edition)
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transgenic rice over-expressing *BvMTSH* show improve drought tolerance without affecting growth.

RESULTS

Transformation of rice with the recombinant fusion gene *BvMTSH*

To introduce the trehalose biosynthesis pathway without producing T6P as an intermediate in plants, we used a gene that encodes for a bifunctional in-frame fusion (*BvMTSH*) of the *BvMTS* and *BvMTH* genes from the nonpathogenic bacterium *Brevibacterium helvolum* (Fig. 1) (15). The *BvMTSH* coding sequences were expressed under the control of the constitutive rice *cytochrome c* promoter (101MTSH) (16) or the ABA-inducible *Ai* promoter (105MTSH) (17). Transgene copy numbers were determined using Southern blotting analysis (data not shown), which revealed that each insertion was independent. Transgene expression levels in 101MTSH and 105MTSH plants were examined using RT-PCR analyses (Fig. 1C). Trehalose contents in 101MTSH and 105MTSH seedlings were measured by gas chromatography–mass spectrometry (GC-MS) (Fig. S1). Trehalose contents of 101MTSH and 105MTSH plants were increased approximately two-fold compared with NT controls. All of the transgenic lines grew without growth inhibition during the vegetative growth stage; T_1 to T_4 seeds were collected from individual transgenic plants, and three independent homozygous T_4 lines for each construct were used for further analysis.

Expression of the *BvMTSH* gene improved drought-stress tolerance

To evaluate the response of 101MTSH and 105MTSH transgenic plants to a water deficit, 4-week-old nontransgenic (NT) control plants and T_4 transgenic seedlings were subjected to drought stress for 2 to 3 days followed by re-watering (Fig. 2A).

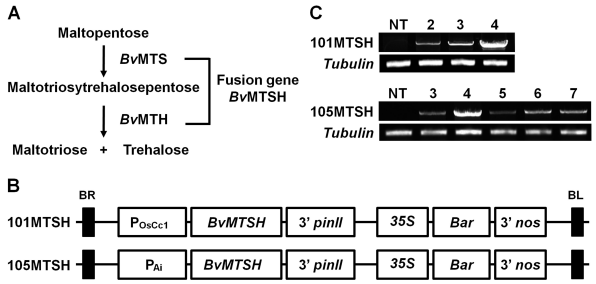

Fig. 1. Production of *BvMTSH* over-expressed transgenic rice plants. (A) Trehalose synthesis pathway (TreY/TreZ) from maltopentaose by *Bv*MTS and *Bv*MTH. *Bv*MTSH is the fusion protein containing the *Bv*MTS and *Bv*MTH proteins. (B) The over-expression plasmids 101MTSH and 105MTSH. (C) Transgene expression levels in 101MTSH and 105MTSH plants were examined using RT-PCR analyses with the total RNA derived from the leaf tissues of 14-day-old seedlings grown under normal conditions.

During re-watering, 101MTSH and 105MTSH plants showed better recovery from the drought-stress test and more growth compared to the severely injured NT plants. In 105MTSH plants, the ability to recover from drought stress was the greatest in line 4, which is consistent with the level of *BvMTSH* expression in the 105MTSH lines (Fig. 1C). After re-watering, 101MTSH showed the highest survival rate (Fig. 2A). The survival rates of the transgenic seedlings were 92-100% for the 101MTSH plants and 63-93% for the 105MTSH plants. These results indicate that over-expression of the *BvMTSH* fusion gene can improve the drought tolerance of transgenic rice.

The drought-stressed plants exhibited visual symptoms with a concomitant loss of chlorophyll. The F_v/F_m is a parameter

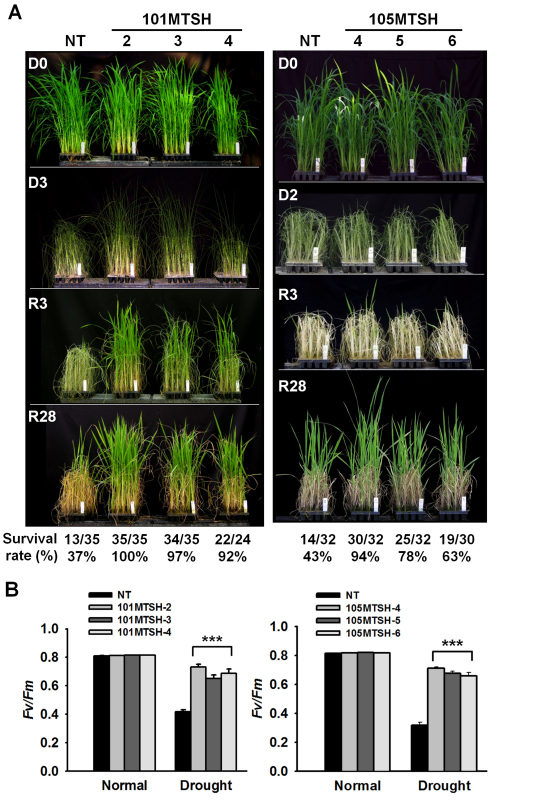

Fig. 2. Drought-stress assays of 101MTSH and 105MTSH transgenic rice. (A) Drought-stress tolerance of 101MTSH and 105MTSH transgenic plants. Three independent homozygous T_4 lines of 101MTSH and 105MTSH and NT controls were subjected to 2-3 days of drought stress followed by re-watering. Pictures were taken at 0, 2 and 3 days after water draining (D0 to D3) and at 3 and 28 days after re-watering (R3 and R28). (B) Changes in the chlorophyll fluorescence (F_v/F_m) of 101MTSH and 105MTSH transgenic plants in response to drought stress. Leaf discs from transgenic and NT plants were used for the experiments. The data represent the means ± SE (n = 9) of three independent experiments. Asterisks indicate statistically significant differences from NT, which was calculated using Student's *t*-test. ***P < 0.001.

widely used to indicate the maximum fluorescence after dark adaptation, which represents the maximum quantum yield of PSII. Healthy plants typically achieve a maximum F_v/F_m value of approximately 0.80, and values lower are observed in plants exposed to abiotic stress factors (18). To further verify the stress tolerance of 101MTSH and 105MTSH transgenic plants, we measured variations in the chlorophyll fluorescence ratio (F_v/F_m) after drought-stress treatments. For the stress treatments, the leaf discs of 3-week-old transgenic and NT seedlings were exposed to drought stress, and the reductions in the F_v/F_m values were measured (Fig. 2B). The values for F_v/F_m were higher by approximately 75% in the 101MTSH and 105MTSH plants compared to the NT control plants under the drought-stress conditions. The results of the stress test experiments confirmed that the 101MTSH and 105MTSH transgenic rice plants presented increased tolerance to drought stress during the vegetative stage. Therefore, the bifunctional in-frame fusion gene *BvMTSH* is useful in stress-tolerant plants that have been genetically engineered to contain the trehalose synthesis pathway.

ABA sensitivity of transgenic plants

Trehalose biosynthesis genes are reported to involve ABA and stress response (19-21). To test this, we analyzed the expression levels of a PP2C family gene (*Abi2*), a SnRK family gene (*SAPK10*), and several ABA/stress induced genes (*LEA3, Rab16, Wsi18, SalT, Dip1, ASR1*) in the transgenic plants under normal condition (Fig. S2). Only the expression level of *ASR1* and *SalT* were commonly increased. We also analyzed the expression levels of ABA biosynthesis genes (*ABA1, ABA2, ABA4, OsNCED1*) (Fig. S3A). The expression level of *ABA2* was down-regulated in the 101MTSH plants. To known the effect of MTSH overexpression on rice trehalose biosynthesis genes, the transcript levels of *OsTPS1* and several *OsTPP* genes were analyzed (Fig. S3B). The expression levels of *OsTPP3* and *OsTPP5* were commonly decreased in the MTSH overexpressed plants. To study the effect of the 101MTSH and 105MTSH constructs on shoot and root growth under ABA conditions, the shoots and roots of the transgenic plants were studied. Seedlings of NT as well as 101MTSH and 105MTSH transgenic plants were grown on half-strength MS solid medium that contained 3 μM of ABA for 7 days (Fig. 3). Under normal conditions, 105MTSH line 4 exhibited shorter shoot lengths and 101MTSH line 4 showed longer root lengths. The other seedlings of the 101MTSH and 105MTSH lines grew at similar rates compared to NT control plants (Fig. 3A and B). In contrast, the root lengths of the 101MTSH and 105MTSH plants were significantly longer than the NT controls under ABA treatment (Fig. 3C and D). These results suggest that 101MTSH and 105MTSH transgenic plants are ABA hyposensitive in the roots.

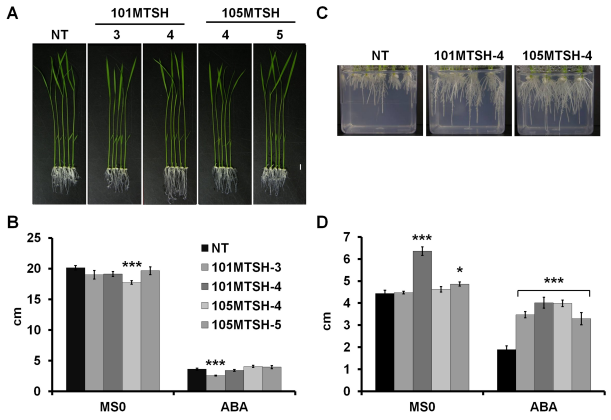

Fig. 3. The effect of the 101MTSH and 105MTSH constructs on growth. The phenotypes of 101MTSH and 105MTSH plants grown under normal (A) and ABA conditions (C). The shoot lengths (B) and root lengths (D) were analyzed under normal and ABA conditions.

DISCUSSION

Trehalose accumulates under abiotic stress in the resurrection plants and in many other plants. Trehalose accumulation under stress is related to the transcriptional activation of the trehalose biosynthesis genes (4). Abiotic stress tolerance has been modulated by engineering the trehalose synthesis pathway in many plants. Unfortunately, many stress-tolerant plants that contain the genetically engineered trehalose synthesis pathway also show abnormal development, such as growth inhibition (8-12). Furthermore, exogenous application of trehalose caused a decrease in NaCl accumulation and growth inhibition in rice (7). In *Arabidopsis*, adding exogenous trehalose induced the expression of genes involved in detoxification, the stress response, and growth inhibition (22). Previously, the mechanism of the undesired side effects of trehalose had been unknown. T6P is believed to be involved in these effects (23). There are several reports that support T6P as a potential agent. T6P accumulation exhibited growth inhibition in *Arabidopsis* (24). T6P has been reported to be a signaling metabolite that is involved in carbon utilization as well as growth and development (13, 23). A model has been proposed where T6P inhibition of SnRK1 is part of a growth-regulating loop in young and metabolically active heterotrophic plant tissues (23).

We hypothesized that trehalose biosynthesis that excludes T6P as an intermediate could lead to improved abiotic stress tolerance in transgenic plants without abnormal growth. There are at least five known biosynthetic pathways for trehalose synthesis (25). To examine this, we used the TreY/TreZ pathway. In this pathway, the biosynthesis of trehalose is mediated by TreY and TreZ without T6P as an intermediate. TreY converts α-1,4-glycosidic linkages at the reducing ends of maltooligosaccharides into α-1,1 linkages, which produces maltooligosyltrehalose. TreZ hydrolyzes the second α-1,4-glycosidic linkage of the intermediate to release trehalose (25). The non-

pathogenic bacterium *Brevibacterium helvolum* contains these two enzymes, *Bv*MTS (TreY) and *Bv*MTH (TreZ) (14). In this study, we used the *BvMTSH* gene (15) that encodes a bifunctional in-frame fusion of the *BvMTS* and *BvMTH* genes of *Brevibacterium helvolum* (Fig. 1). The fusion enzymes has some advantages, such as simple expression of a single recombinant gene and faster rates of sequential enzyme reactions by facilitating transfer of reaction intermediates to the catalytic sites of the next enzymes. In addition, the recombinant enzymes can produce trehalose from soluble starch without α-amylase (15). We produced 101MTSH and 105MTSH transgenic plants over-expressed *BvMTSH* under the control of the constitutive rice *cytochrome c* promoter or the ABA-inducible *Ai* promoter, respectively.

101MTSH and 105MTSH plants displayed significantly enhanced drought-stress tolerance (Fig. 2), which is in agreement with previous reports where trehalose synthesis was engineered into plants. For example, drought tolerance was obtained by over-expression of the yeast *ScTPS1* gene in tobacco (8), the *AtTPS1* gene in *Arabidopsis* (26), and the plastid *TPS1* gene in tobacco (27). Over-expression of the bifunctional fusion genes *OtsA-OtsB* and *ScTPS-ScTPP* exhibited high trehalose accumulation and improved abiotic stress tolerance in rice and tobacco, respectively (27, 28). The trehalose level increased three to four-fold after drought stress in tobacco (27). In *Arabidopsis*, heat and cold stress lead to two- and eight-fold increase of the trehalose level, respectively (29). Moreover, the trehalose content in *OsTPS1* over-expressed transgenic rice which exhibit enhancement of abiotic stress tolerance was increased 1.45 to 2.01-fold than wild-type (21). GC-MS analysis showed that trehalose contents of 101MTSH and 105MTSH plants were increased approximately two-fold compared with NT controls. These results suggest that over-expressing *BvMTSH* enhanced the tolerance of rice seedling to drought by increasing trehalose levels. Furthermore, 101MTSH and 105MTSH plants exhibited no abnormal plant development or visible phenotypic alterations during vegetative growth. Under normal conditions, 105MTSH line 4 exhibited shorter shoot lengths and 101MTSH line 4 showed longer root lengths (Fig. 3). Even though, theses two lines showed higher trehalose content than other lines, the difference is about 25%. Moreover, 105MTSH line 4 and 101MTSH line 4 showed phenotype in different tissues, shoot and root, respectively. Therefore, we think that these variations could be resulted from transformation effect, such as somatic variation or transgene position effect. Moreover, 101MTSH and 105MTSH plants also grew normally and matured in the paddy field (data not shown). Our results suggest that the *BvMTSH* bifunctional in-frame fusion gene is useful in the production of stress-tolerant plants that show no growth inhibition when they have been genetically engineered to contain the trehalose synthesis pathway.

ABA is an important plant hormone in the abiotic stress response. Abiotic stresses, such as drought, trigger the ABA signaling pathway. Many molecular and cellular responses, including the expression of stress-related genes, are initiated by the ABA signaling pathway. Trehalose biosynthesis genes are reported to be involved in ABA signaling and stress response (19-21). The expression of *AtTPS1* results in an ABA-insensitive phenotype in *Arabidopsis* (26). In rice, two *TPP* genes are transiently induced by drought and exogenous ABA application in seedling roots and shoots (30). To investigate the relationship between trehalose-induced drought-stress tolerance and ABA signaling, 101MTSH and 105MTSH transgenic seedlings were grown under 3 μM ABA conditions. In response to ABA treatment, the shoot lengths of 101MTSH and 105MTSH seedlings showed no significant difference, except 101MTSH line 3. In contrast, the root lengths of 101MTSH and 105MTSH seedlings showed an ABA hyposensitive phenotype (Fig. 3B). This result indicates that trehalose is involved in ABA response in the roots, and over-expression of *BvMTSH* reduced root growth inhibition by ABA in rice.

In general, stress-inducible promoters have been considered the better promoters for transgenic plants enhanced stress tolerance than constitutive promoters. Even though, 101MTSH showed higher survival rate than 105MTSH (Fig. 2A), stress inducible promoter showed low changes to growth alteration. These results suggest that ABA-inducible promoter (Ai promoter) is more suitable for the development of drought resistant transenic rice using *BvMTSH* gene than constitutive promoter (cytochrome c promoter). This study indicates that the over-expression of *BvMTSH* enhanced drought-stress tolerance without any abnormal growth. Moreover, trehalose is involved in ABA response in the roots. Our results suggest that the *BvMTSH* bifunctional in-frame fusion gene is useful in the production of stress-tolerant plants genetically engineered for the trehalose synthesis pathway.

MATERIALS AND METHODS

Plasmid construction and transformation of rice

Transgenic and non-transgenic (NT) rice plants with an *Oryza sativa* subsp. *japonica* cv. Nakdong background were used. The complete fusion gene *BvMTSH* was used. The *att*B-PCR products of *BvMTSH* using the primers MTSH-ATG (5′-AAAAAGCAGGCTCATGAAGACTCCGGTCTCCAC) and MTSH-TGA (5′-AGAAAGCTGGGTGCCGGATCAAGCTTCAGGACT) were inserted into pMJ101 and pMJ105 through BP- and LR-recombination reactions performed according to the manufacturer's instructions (Invitrogen). The over-expression plasmids pMJ101 and pMJ105 contained the constitutive rice *cytochrome c* promoter (101MTSH) or ABA-inducible *Ai* promoter (105MTSH), respectively. The plasmids were introduced into *Agrobacterium tumefaciens* LBA4404 by triparental mating (31).

Semiquantitative reverse transcription (RT)-PCR

Total RNA was isolated from the rice tissue samples using the

TRI REAGENT® (Molecular Research Center) according to the manufacturer's instructions. For RT-PCR, the first strand of cDNA was synthesized from 5 μg of total RNA as a template using oligo $(dT)_{18}$ primers according to the manufacturer's instructions (RevertAid™ First Strand cDNA Synthesis Kit, Fermentas). A one-third dilution of the cDNA synthesis reaction mixture was prepared, and 1 μl of the diluted cDNA mixture was used as a template (32). The primers MTSH-ATG and MTSH-in1R (5′-ACGTCAGCCAGTGCGTCGTA) were used for the PCR. PCR was performed at 95°C for 10 min, followed by 22 to 32 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 30 s. Amplified products were resolved on a 1.5% (w/v) agarose gel. *Tubulin* was used as reference gene (tubulin-F, 5′-CATCGACATCAAGTTCGACC; tubulin-R, 5′-TC ACCATCGT CGAACTCGGA). The same results were obtained in three independent experiments. However, only the result from one experiment is presented.

Drought stress, ABA treatment and chlorophyll fluorescence measurements

For ABA treatment, independent homozygous T_4 lines of 101MTSH and 105MTSH transgenic and NT control seeds were germinated on MS solid medium for 2 days. After germination, germinants of equal size were selected and transferred to MS solid medium containing 3 μM of ABA. The germinated seedlings were incubated with 16 h light/ 8 h dark cycles at 28°C in a growth chamber. To test drought-stress resistance, four-week-old NT and transgenic plants grown on soil were subjected to 2-3 days without water followed by watering in a greenhouse (33). The chlorophyll fluorescence of three-week-old NT and transgenic plants was measured using a pulse modulation fluorometer (mini-PAM, Walz, Germany). For the leaf disc test, the green portions of approximately 10 seedlings were cut using scissors prior to stress treatments *in vitro*. Under continuous light at 150 μmol m^{-2} s^{-1}, the leaf discs were air-dried for 2 h (to induce drought stress). After drought-stress treatment, the leaf discs were dark-adapted for 10 minutes, and the minimal fluorescence level (F_0) was measured; a saturating light pulse was applied, and the maximal fluorescence level (F_m) was measured. The ratio of F_v to F_m (F_v/F_m = F_m-F_0/F_m), which represents the activity of photosystem II, was used to assess the functional damage to the plants (34). The statistical significance of differences between groups was assessed using Student's *t*-test.

Acknowledgements

We thank Baek Hie Nahm and Ju-Kon Kim at Myongji University for their helpful advice. This work was supported by the Technology Development Program for Life Industry through the Korea Institute of Planning and Evaluation for Technology of Food, Agriculture, Forestry and Fisheries (grant number 111076-5).

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