Mitochondrial small heat-shock protein enhances thermotolerance in tobacco plants

Kazutsuka Sanmiya, Katsumi Suzuki, Yoshinobu Egawa, Mariko Shono*

Okinawa Subtropical Station, Japan International Research Center for Agricultural Sciences, Ishigaki, Okinawa 9070002, Japan

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Abstract To clarify the role of mitochondrial small heat-shock protein (MT-sHSP) in the heat-shock response, we introduced the tomato (*Lycopersicon esculentum*) MT-sHSP gene under the control of the 35S promoter into tobacco (*Nicotiana tabacum*), and examined the thermotolerance of the transformed plants. Irrespective of the orientation, sense or antisense, of the gene, the transgenic plants exhibited a normal morphology and growth rate in the vegetative growth stage. When 4-week-old seedlings were exposed to sudden heat stress, the sense plants which overexpress the MT-sHSP gene exhibited thermotolerance, whereas the antisense plants in which the expression of the gene is suppressed exhibited susceptibility.

Key words: Mitochondrial small heat-shock protein; Thermotolerance; Tobacco; Transformation

1. Introduction

All organisms have evolved mechanisms to overcome environmental stress. Such mechanisms are thought to be especially important in plants, which have the disadvantage of immobility. Although severe heat stress leads to irreversible damage or cell death, a sublethal dose induces the heat-shock response, which leads to a higher level of acquired thermotolerance [1]. Once organisms have acquired a higher level of thermotolerance, they can endure lethal heat stress to some extent and resume normal cellular activities, when the stress ends. A previous study suggests that heat-shock proteins (HSPs) are the essential components of the heat-shock response [2].

HSPs are classified based on molecular weight (HSP100, 90, 70, and 60, and small HSPs (sHSPs)). sHSPs are further classified based on their localization in the cell, i.e. the cytoplasm, nucleus, mitochondria, chloroplasts or endoplasmic reticulum [3]. They share a common primary structure in their C-termini that shows high homology to α-crystallins [4]. The diversity of sHSPs is specific to plants, since other eukaryotes have far fewer sHSPs [5].

Previous studies have revealed that sHSPs act as molecular chaperones [6–11]. A few sHSPs have been shown to bind thermally denatured proteins at their surface to maintain a folding-competent state [6,9], and a model was proposed in which sHSPs act as a reservoir of partly folded proteins accessible to refolding by other molecular chaperones [12]. However, the roles of sHSPs in the molecular mechanism of the heat-shock response in vivo are mostly unknown.

Plants synthesize predominantly sHSPs during the heat-shock response [13], and it was indicated that the accumulation of HSP25 is correlated with thermotolerance [14]. In addition, a gene for rice, Oshsp16.9, conferred thermotolerance upon *Escherichia coli* [15], and the altered expression of a gene for carrot Hsp17.7 correlated with the thermotolerance of transformed carrot plants [16]. These results suggest that the sHSPs play an important part in thermotolerance.

The breakdown and abnormal metabolic activities of mitochondria lead to a decrease in cell viability under heat stress. In addition, the accumulation of mitochondrial (MT) sHSP was synchronized with the thermotolerance of mitochondria [17]. These findings raise the question of whether MT-sHSP confers thermotolerance upon mitochondria and subsequently upon the cell and individual plant.

We have demonstrated that tomato MT-sHSP has a molecular chaperone function in vitro [10]. In this study, we have transformed tobacco with the tomato MT-sHSP gene to determine the role of MT-sHSP in the heat-shock response of plants.

2. Materials and methods

2.1. Plant material

Tobacco wild type and transgenic plants (*Nicotiana tabacum* L. cv. SR1 and SR1 carrying the MT-sHSP gene, respectively) were germinated and grown for 4 weeks on Murashige and Skoog (MS) medium in a growth chamber at 25°C. Then, they were transferred to soil for further cultivation to observe their morphology and growth in a greenhouse at 25°C.

2.2. Vector construction and transformation of tobacco plants

Following deletion of the β-glucuronidase gene from pBI121, a plant transformation vector, the full-length tomato MT-sHSP cDNA was subcloned into the vector to make sense (pBInMTsHSP) and antisense (pBInRVMTsHSP) constructs. These vectors, pBI121, pBInMTsHSP and pBInRVMTsHSP, were introduced into *Agrobacterium tumefaciens* LBA4404 by the freezing transformation method [18]. The resultant vector construction in *A. tumefaciens* was confirmed by the plasmid preparation from *A. tumefaciens* and restriction analysis of the plasmid.

Leaf disk transformation using SR1 plants was performed as described previously [19]. Disks infected with *A. tumefaciens* were incubated on medium for inducing shoots. After a few weeks, the regenerated shoots were transferred to medium for inducing roots. Both media contained carbenicillin (500 μg/ml) and kanamycin (100 μg/ml).

*Corresponding author. Fax: (81)-980-88 6204.
E-mail address: mshono@jircas.affrc.go.jp (M. Shono).

Abbreviations: GST, glutathione *S*-transferase; MS, Murashige and Skoog; MT-sHSP, mitochondrial small heat-shock protein

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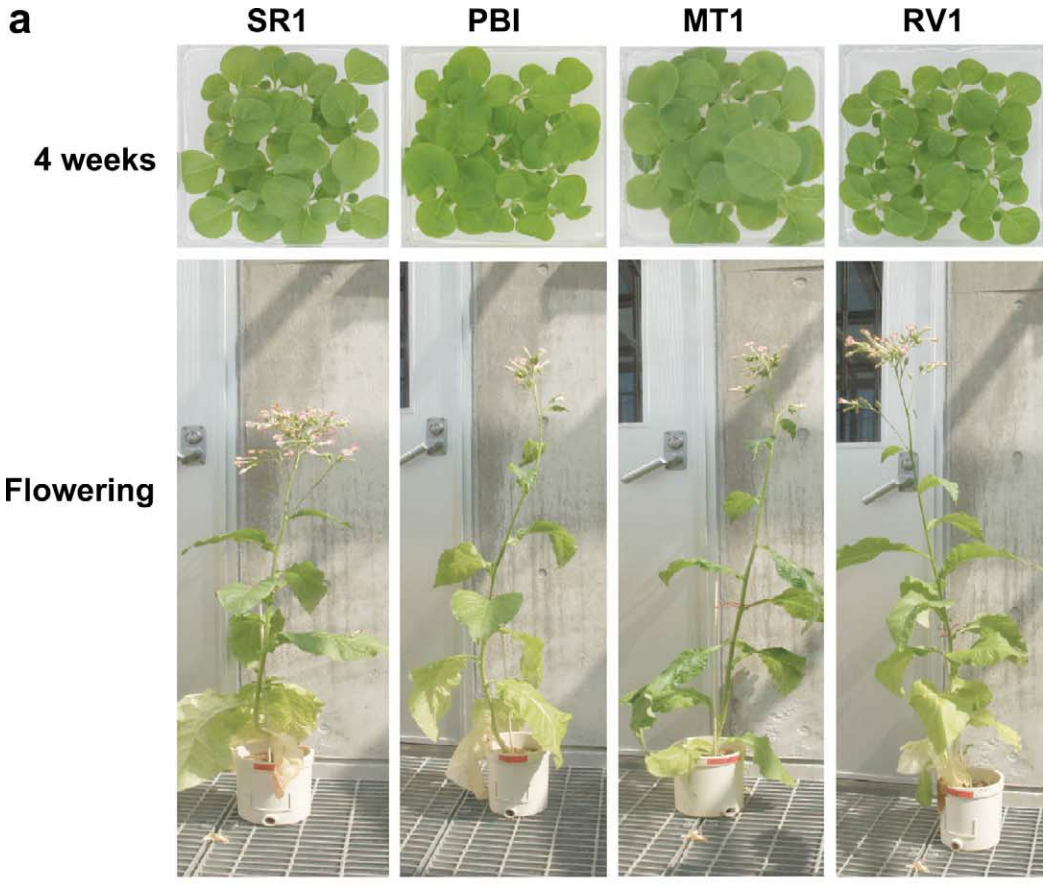

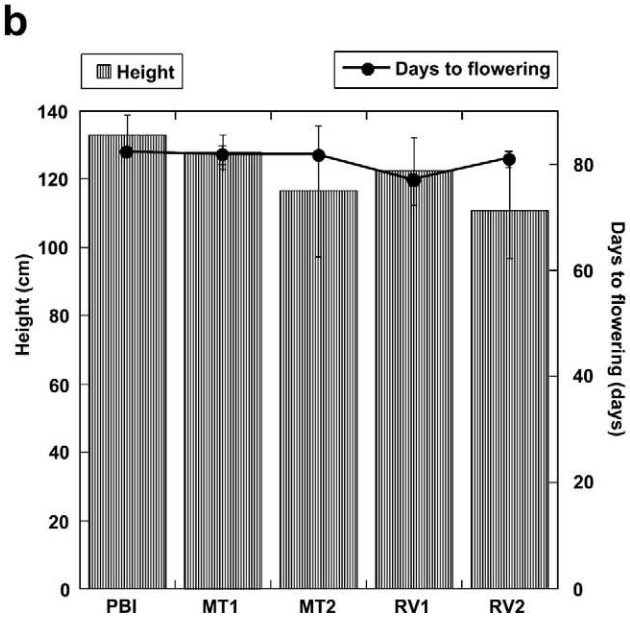

Fig. 1. Transgenic tobacco plants with the tomato MT-sHSP gene. Tobacco wild type, SR1 and T_2 transgenic lines into which were introduced the construct without an insert (PBI), with a sense-oriented MT-sHSP gene or with an antisense-oriented gene (MT1 and RV1, respectively) were germinated and grown on MS medium for 4 weeks. Then the young seedlings were transferred to soil in a greenhouse and kept at 25°C. a: The morphology and growth rate of the plants were observed 4 weeks after the seeding and on the day of flowering. b: The height on the day of flowering was measured and the number of days until flowering was recorded. Results represent the average from measurements of five individuals.

T_0 transgenic plants were screened using kanamycin with the marker on the vector from shoots generated by the incubation of transformed tobacco leaf disks. T_0 plants were further used to obtain T_1 and T_2 lines that were confirmed to be homozygous by the germination rate of their seeds (T_2 and T_3) on kanamycin-containing medium and by their thermotolerance (data not shown). As a consequence, one control line, three sense lines and two antisense lines were obtained. Each transgenic line seems to represent an independent integration event since in each line a specific DNA fragment was observed by genomic Southern blotting analysis (data not shown).

2.3. Thermotolerance assay

Plants of the wild type and T_2 homozygous lines with the control, sense or antisense construct were used for the thermotolerance assay. Using a growth chamber (FLI-301N, Eyela, Japan), 4-week-old plants grown at 25°C were incubated at 46°C or 48°C for 2 h, then further incubated at 25°C in a greenhouse for 7 days. Thermotolerance was assessed based on visual observation.

2.4. Polyclonal antibody

The full-length cDNA for the tomato MT-sHSP gene was subcloned into the glutathione *S*-transferase (GST) vector (Amersham), an expression vector in *E. coli*. The GST-MT-sHSP fusion protein was purified according to the instruction manual and used as the antigen. Rabbit anti-MT-sHSP antibody was produced and affinity-purified by Sawady technology.

2.5. Immunoblotting

Four-week-old tobacco plants were homogenized in the protein extraction buffer (50 mM Tris–HCl pH 7.5, 0.25 M sucrose, 5 mM dithiothreitol, and 1 mM EDTA), then the extract was centrifuged at $3000\times g$ for 10 min. The supernatant was further centrifuged at $10\,000\times g$ for 10 min. The precipitate of the $10\,000\times g$ centrifugation was used as the mitochondrial fraction for immunoblotting. Twenty micrograms of protein was electrophoresed, then using anti-MT-sHSP antibody, immunoblotting was performed according to the instructions for the Vectastain ABC KIT (Vector Laboratories, Burlingame, CA, USA).

3. Results and discussion

3.1. Morphology and growth rate of the transgenic plants

Genes for MT-sHSP have been cloned from several plant species [10,20–24], and previous studies reported the altered expression of sHSPs in plants [16,25,26]. However, there has been no report that the expression of MT-sHSP was altered. To investigate the role of MT-sHSP, we introduced the gene for MT-sHSP into tobacco plants.

Some classes of sHSPs are known to be expressed under normal growth conditions and during development [27–30]. Thus, it was suggested that sHSPs play roles in housekeeping and development, in addition to the heat-shock response. Nevertheless, the role of MT-sHSP in housekeeping and development is yet to be clarified. In our study, irrespective of the vector construct, all of the transgenic plants screened exhibited a morphology and growth rate similar to wild type plants at the young seedling stage and on flowering (Fig. 1) and throughout their life cycle (data not shown). Consequently, the introduced MT-sHSP did not seem to influence the development of the transgenic plants. It is suggested that MT-sHSP plays an important role specifically in the heat-shock response.

3.2. MT-sHSP expression in the transgenic plants

To assess the expression of the gene for MT-sHSP in screened plants of the T_2 lines, the 4-week-old seedlings were subjected to Western blot analysis using anti-MT-sHSP antibody. In the seedlings of the wild type and control line,

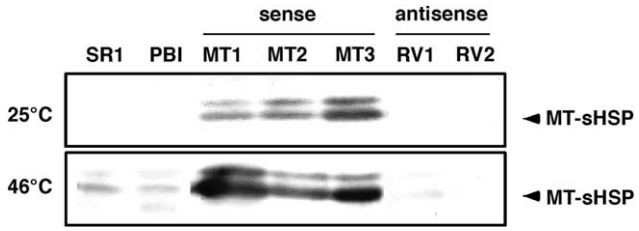

Fig. 2. Expression of the gene for MT-sHSP in the transgenic plants. Four-week-old seedlings of the wild type (SR1) and T_2 homozygous lines into which was introduced a vector without an insert (PBI) or the MT-sHSP gene (sense: MT1, 2 and 3, antisense: RV1 and 2) were subjected to Western blot analysis using anti-MT-sHSP antibody. Mitochondrial fractions for the analysis were prepared using seedlings sampled before (25°C) and after (46°C) the heat treatment for 2 h.

MT-sHSP was not detected at 25°C but accumulated after sudden heat stress treatment at 46°C for 2 h (Fig. 2). Therefore, the transformation by itself did not affect the expression of the gene for MT-sHSP in the tobacco genome. In the seedlings of the sense lines, MT1, MT2 and MT3, the accumulation of MT-sHSP occurred even at 25°C and it was abundantly observed after the heat stress (Fig. 2). In the seedlings of the antisense lines, RV1 and RV2, MT-sHSP was not detected at 25°C (Fig. 2) as in the wild type and control line. Although MT-sHSP was barely detectable in RV1 seedlings following heat stress, the responsiveness of the endogenous tobacco gene for MT-sHSP was markedly reduced in both antisense lines, RV1 and RV2, in comparison with the wild type and vector control lines (Fig. 2).

The accumulation of MT-sHSP in the seedlings of the wild type and vector control lines is thought to be caused by the expression of the corresponding tobacco gene. Thus, the anti-MT-sHSP antibody seems to have the ability to react with the tobacco MT-sHSP, although it was raised against the recombinant tomato MT-sHSP. In addition to the mature MT-sHSP (Fig. 2, MT-sHSP), immature MT-sHSP with the transit peptide was also likely to react with the antibody, because a signal for the molecular mass calculated with the primary structure of the protein was observed (Fig. 2).

3.3. Thermotolerance of the transgenic plants

Although the treatment at 46°C for 2 h resulted in no or little damage to the seedlings of the wild type, vector control and sense lines, it resulted in severe damage to the antisense seedlings (Fig. 3). Treatment at 48°C for 2 h resulted in severe damage to the seedlings of the wild type, vector control and antisense lines, but only weakly affected the survival of the seedlings of the sense lines (Fig. 3). Also, while treatment at 44°C resulted in no apparent reduction in viability, treatment at 50°C was lethal to seedlings from all lines (data not shown).

As expected, the thermotolerance of the vector control seedlings was equal to that of the wild type seedlings. These results seem to be caused by the similar expression profile of the gene for sHSPs in the two lines.

Acquired thermotolerance is defined as the ability induced by a sublethal heat stress to overcome subsequent exposures to lethal high temperatures [1]. Actually, the sublethal treatment at 40°C for 2 h followed by the severe heat stress at 48°C for 2 h led to acquired thermotolerance in the wild type and vector control tobacco plants in our experiments (data not shown). Meanwhile, Downs et al. revealed that the heat

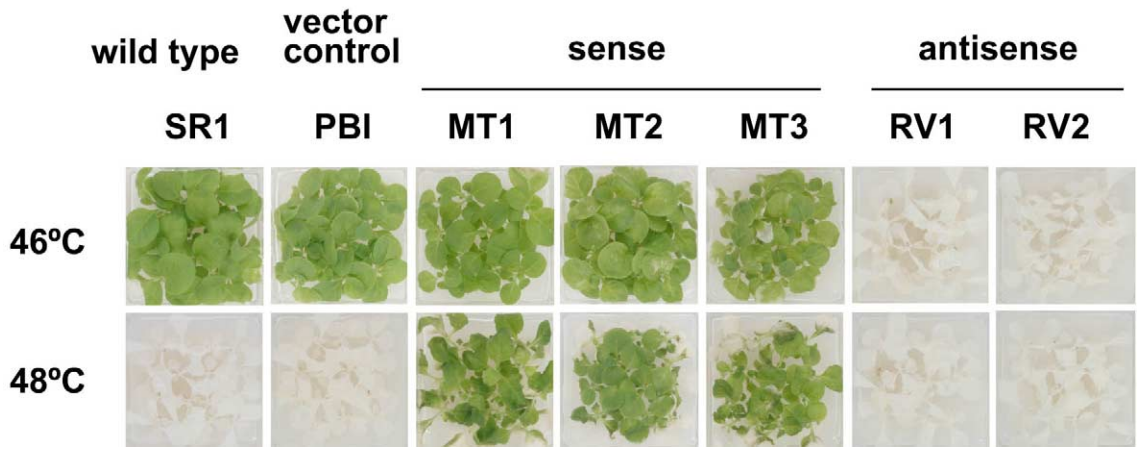

Fig. 3. Thermotolerance of the transgenic plants with the MT-sHSP gene. Four-week-old seedlings of the wild type and T_2 homozygous lines grown as mentioned in Fig. 2 were exposed to heat stress at 46°C or 48°C for 2 h. The seedlings were photographed 7 days after the treatment.

acclimation of mitochondrial electron transport is accounted for by the protective function of MT-sHSP under heat stress [31]. As the mitochondrion is an essential organelle, it is possible that the heat acclimation of mitochondria leads to the thermotolerance of individuals. The thermotolerance of the sense line seedlings in this study supports this hypothesis. The tolerance of the sense plants may be considered to be a kind of acquired thermotolerance, because MT-sHSP accumulated even under normal temperature conditions as if the seedlings were exposed to a sublethal heat stress.

Nevertheless, the question is raised as to whether MT-sHSP plays an important role in heat-shock response or not, since plants utilize many classes of sHSPs. In our study, under sublethal conditions, most of the sHSPs encoded by the tobacco genome should have been expressed in the wild type and control line. Meanwhile, the similarity of primary structure is not particularly high between the gene for MT-sHSP and the genes for other sHSPs [10]. Thus, in the antisense lines, the introduced gene is not likely to inflict on the gene expression for sHSPs except MT-sHSP. In other words, under heat stress, in the antisense lines, it is suggested that the introduced antisense MT-sHSP gene only suppresses the endogenous tobacco MT-sHSP gene, and other sHSPs are induced by the stress. Consequently, the reduction in MT-sHSP expression seems to lead directly to susceptibility to heat stress. Considering this, MT-sHSP is suggested to have a pivotal role in the response to heat shock in tobacco plants.

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