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MOLECULAR CHARACTERIZATION OF A STRESS-RELATED GENE *MsTPP* IN RELATION TO SOMATIC EMBRYOGENESIS OF ALFALFA

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Abstract

Somatic embryogenesis is the most comprehensive form of cell totipotency in plant tissue culture *İn vitro*, in which plant growth regulator and stress have been regarded as the important factors. In the present work, a full-length cDNA of stress-related trehalose-6-phosphate phosphatase, designated as *MsTPP*, was isolated and characterized from the non-embryogenetic callus of alfalfa on the medium involved in thidiazuron (TDZ). Homology analysis showed that MsTPP protein contained two conversed phosphatase boxes, which is the typical characterization of all phosphatases. Semi-quantitative RT-PCR and real-time PCR demonstrated that *MsTPP* was expressed specifically in young root of alfalfa seedling and up-regulated by TDZ during the whole stage of somatic embryogenesis of alfalfa. Furthermore, treatments with 0.93 or 50 μM TDZ strongly suppressed embryogenic competence of the callus, while increased transcript levels of *MsTPP*. Taken together, these results suggest that *MsTPP* is likely to be associated with obtaining of embryogenetic competence in alfalfa. This study expands our understanding of the molecular mechanism of TDZ on reduction or inhibition of the embryogenic competence of alfalfa callus.

Introduction

Somatic embryogenesis is the developmental process whereby somatic cells undergo a series of morphological and biochemical changes to differentiate into somatic embryos and ultimately into plants which resemble the zygotic stages of development *İn vitro* (Dodeman *et al*., 1997; Schmidt *et al*., 1997). In higher plant, somatic embryogenesis is a typical expression of totipotency. According to the report of Namasivayam (2007), the whole process of regeneration of plant through somatic embryogenesis could be divided into two stages: induction and expression of embryogenic competence. It has been thought as a key step that embryogenetic competence of somatic cells acquires and maintenances in somatic embryogenesis (Namasivayam, 2007). Although more and more studies have showed that somatic embryogenesis widely exists in dicot and monocot plants, much less is known about the processes that somatic cells transfer into the embryogenic cells and form the somatic embryos under suitable culture conditions (Yang *et al*., 2010).

Plant growth regulators play a central role in the process of somatic embryogenesis, which act as the main factor to regulate the changes of embryogenic competence in somatic cells of explants (Fehér *et al*., 2003). Somatic embryogenesis is an extreme stress response of cultured plant cells *İn vitro* and embryogenic competence of somatic cells in the explants can be triggered by different stress factors, such as osmotic pressure, heavy metal ions, high/low temperature, pH, dehydration and explant wounding (Dudits *et al*., 1991; Kiyosue *et al*., 1993; Karami & Saidi, 2010; Rafique *et al*., 2011). Thus, stress has been considered to be another signal factor that somatic cells could dedifferentiate and obtain embryogenic competence in the process of somatic embryo induction (Lee *et al*., 2001; Ikeda-Iwai *et al*., 2003; Malabadi & van Staden, 2006; Lincy *et al*., 2009). Many stress-related genes and proteins related with somatic embryogenesis, such as CaM-related genes and heat shock proteins have been isolated and identified to (Karami & Saidi, 2010). Moreover, in early development of somatic embryogenesis of banana, high levels of antioxidant enzyme activity and high H_2O_2 content play an important role for embryogenic competence (Ma *et al*., 2012). However, little attention has been paid to how plants actually responding to stressful conditions through the functions of certain genes in the early events of somatic embryogenesis.

Our previous studies have showed that embryogenic competence of callus induced from alfalfa (*Medicago sativa* L. cv. Jinnan) petioles on B5h medium was inhibited when the kinetin in the medium was replaced by thidiazuron (TDZ) (Huang *et al*., 1994; Zhang *et al*., 2006; Hu *et al*., 2011; Feng *et al*., 2012). We have obtained one Expressed Sequence Tag (EST) (clone QB7) encoding trehalose-6-phosphate phosphatase, termed *MsTPP* gene from the differentially expressed ESTs during alfalfa callus formation under TDZ-treated condition (Zhang *et al*., 2006). TPP is one of the key enzymes in the trehalose biosynthetic pathway in higher plants (Avonce *et al*., 2006). Trehalose has commonly been found to be function as a reserve carbohydrate and stress protectant in a wide range of organisms, including bacteria, fungi and invertebrates (Elbein *et al*., 2003). In higher plants more evidences have suggested an important regulatory function for trehalose metabolism in plant growth, stress tolerance, photosynthetic activity and carbon partitioning (Elbein *et al*., 2003). Overexpression of trehalose synthesis genes can be used to improve the abiotic stress tolerance in some plants, such as tobacco, potato, *Arabidopsis*, alfalfa and rice (Romero *et al*., 1997; Yeo *et al*., 2000; Jang *et al*., 2003; Miranda *et al*., 2007; Ge *et al*., 2008; Suárez *et al*., 2010). However, its involvement in somatic embryogenesis is largely unknown.

Therefore, to determine the possible effect of the stress-related gene *MsTPP* in cultured plant cells during the transition of embryogenic competence of TDZ-treated callus in early development of somatic embryogenesis of alfalfa, the isolation and characterization of *MsTPP* from TDZ-treated callus in somatic embryogenesis of alfalfa (*Medicago sativa* L. cv. Jinnan) were described. Moreover, the expression patterns of *MsTPP* gene in various tissues of alfalfa shoot and in cultures treated by TDZ during each

process of somatic embryogenesis, especially in the maintenance phase of alfalfa embryogenic callus under the culture conditions treated with different concentrations of TDZ, were analyzed. Our study expands our understanding of the molecular mechanism of TDZ on reduction or inhibition of the embryogenic competence of alfalfa callus.

Materials and Methods

Somatic embryogenesis and TDZ treatment: Alfalfa (*Medicago sativa* L. cv.Jinnan) plants were grown in green house. Somatic embryos were produced following the methods described by Zhang *et al*., (2006). Briefly, the process of the somatic embryogenesis was divided into 4 phases including callus induction, callus maintenance, somatic embryo induction and somatic embryo maturation. For callus induction, sterilized petioles as the initial explants were incubated for 28 days on B5h medium (Allfrey & Northcote, 1977) containing 4.52 μM 2, 4-D, 0.93 μM kinetin and 30 g l^{-1} sucrose solidified with 7 g l^{-1} agar. For somatic embryos induction, after the induced callus was maintained on the same medium mentioned as above for 28 days, suspension cultures were initiated from the calli in liquid B5g medium (Gamborg *et al*., 1968) supplemented with 4.52 μM 2, 4-D, 0.54 μM NAA and 30 g l^{-1} sucrose for 7 days in an incubated shaker at 100 rpm. Then suspension cultures were sequentially filtered through 500 mm and 230 mm sieves, and the fraction separated on the 230mm sieves were transferred to hormone-free liquid SH medium (Schenk & Hildegrandt, 1972) containing 30 mM proline, 10mM $(NH_4)_2SO_4$ and 30 g l^{-1} maltose for the development of somatic embryos. After 14 days, the number of somatic embryos with two polarities developed was counted to quantify the formation of somatic embryos.

For TDZ treatment, 0.93μM TDZ was added into the medium used in each phase of somatic embryogenesis. The cultures were sampled on the end of each phase, including callus induction for 28 days, callus maintenance for 28 days, somatic embryo induction for 7 days and somatic embryo maturation for 14 days. In addition, TDZ with 0.93 and 50 μM concentrations was employed to replace kinetin in the callus maintenance medium B5h, named as TDZ-0.93 and TDZ-50, respectively. One part of calli was frozen after subculture of 2, 10 and 28 days with or without TDZ treatment in liquid nitrogen for further experiment, and another part was sequentially cultured according to the above for counting the number of somatic embryos.

Isolation of the full length of *MsTPP* using RACR-PCR: Total RNA was extracted from alfalfa callus induced on TDZ-0.93 medium for 28 days using the TRIzol reagent (Invitrogen). The 3'-Full RACE Core Set Kit (TaKaRa) and BD SMART™ RACE cDNA Amplication Kit (BD Biosciences) were respectively used for rapid amplification of 3' and 5' cDNA ends according to manufacturer's instructions. For 3'-RACE, the first gene-specific primer TPP-3 (CGCAATAGGAGCCAAGGGTGTGGGATT) and the nested gene-specific primer TPP-3N (GTCAAGGGGTAGTTCAATGG) were designed from alfalfa EST clone QB7 reported (Zhang *et al*., 2006). For 5'-RACE, the first gene-specific primer TPP-5 (GAATCCCACACCCTTGGCTCCTATTGC) and the nested gene-specific primer TPP-5N (GCAAATCCAAGTGACTCTAGC) were used according the known sequence. The PCR purified product was cloned into pMD-20T vector (TaKaRa) and sequenced by Sangon Company (Shanghai, China) using an ABI 3770 DNA sequencer.

Sequence alignment and phylogenetic analysis: 5' and 3' overlapping sequences were assembled using DNAMAN software (Lynnon Biosoft Company, USA) to obtain full-length sequence. Open reading frame (ORF) and protein prediction were made using NCBI ORF Finder (http://www.ncbi.nlm.nih.gov/). Multiple sequence alignment was performed with the deduced protein sequence and the deposited functional TPP protein sequences of four species in the NCBI protein database, using the Clustal X software (version 1.83). Phylogenetic analysis of these sequences was constructed with the MEGA 4.0 program (Tamura *et al*., 2007).

Semi-quantitative RT-PCR: Total RNA was extracted using TRIzol reagent from root, stem, leaf and petiole of alfalfa shoot. First strand cDNA was synthesized using the Reverse Transcriptase M-MLV (RNase H^-) (TaKaRa) and subsequently used as template for RT-PCR following the manufacturer's protocol. The specific primers MsTPP-UP (CGTTGACGAAAAGAGTTGGG) and MsTPP-DW (CCATTGAACTACCCCTTCACC) were designed based on the *MsTPP* sequence and used to study the expression pattern of *MsTPP* gene in various organs of alfalfa shoot. As internal control, the primers 18S-F (TATGGTTCCTTTGGTCGCTC) and 18S-R (CTTGGATGTGGTAGCCGTTT) were used to amplify a fragment within *18s rRNA* gene from the conversed sequence in dicots. Experiment was repeated three times with similar results.

Real-time PCR: Using TRIzol reagent, total RNA was isolated from frozen cultures described as above, which included a DNase I treatment to remove any DNA contamination. Based on the sequence of *MsTPP* gene, specific primers were designed, namedly MsTPP-F: 5'-TTCCCACAGCCATTGTTAGT-3' and MsTPP-R: 5'-TCTCCTATTTGTTGGACCCT-3'. Expression of *ACTIN* gene (AA660796) was used as internal control (ACTIN-F: 5'-GACAATGGAACTGGAATGG-3' and ACTIN-R: 5'-CAATACCGTGCTCAATGG-3'). Real-time PCR was performed on each cDNA dilution using SYBR GREEN I (TaKaRa) and analyzed in the iCycler IQ real-time PCR detection system (Bio-Rad) according to the manufacture's protocol. Three independent assays were carried out. The results were analysed using the $2^{-\Delta\Delta Ct}$ method (Kenneth & Schmittgen, 2001).

Results and Discussion

Isolation and characterization of *MsTPP*: The complete sequence of *MsTPP* gene was obtained from alfalfa callus induced on TDZ-0.93 medium for 28 days. The full-length *MsTPP* cDNA contained an ORF of 1,140 bp with a 5'-untranslated region of 128 bp and 3'-untranslated region of 364 bp terminated by a string of A residues. The

MsTPP gene encoded a putative polypeptide of 379 amino acids with a predicted molecular weight of 43.02 kDa and an isoelectric point of 9.35. The sequence had been submitted to the GenBank (accession number HM156211).

The putative MsTPP protein showed significant sequence similarity to TPPs that have been reported in different plant species. As shown in Fig. 1, alignment of the deduced amino acid sequence of *MsTPP* with those of *TPP* gene products from *Arabidopsis* (*AtTPPA* and *AtTPPB*), *Oryza sativa* (*OsTPP1*) and *Zea mays* (*ZmRA3*) reveals that *TPP* sequences are moderately conserved with the exception of the N-terminal region (Vogel *et al*., 1998; Pramanik & Imai, 2005; Satoh-Nagasawa *et al*., 2006). In particular, two distinct regions (BOX I and BOX II, Fig. 1), which are unique features of phosphatases and are known as phosphatase boxes (Vogel *et al*., 1998; Habibur *et al*., 2005), are highly conserved with a sequence similarity of more than 90%. A phylogenetic tree was drawn with the sequences of TPP homologues from Arabidopsis, rice, poplar and maize according to reports (Fig. 2) (Li *et al*., 2008). Results showed that there are at least two main groups within the TPPs and the MsTPP had a closer relationship to AtTPPB and ZmRA3 within the same group, suggesting that MsTPP might play a similar role with AtTPPB and ZmRA3.

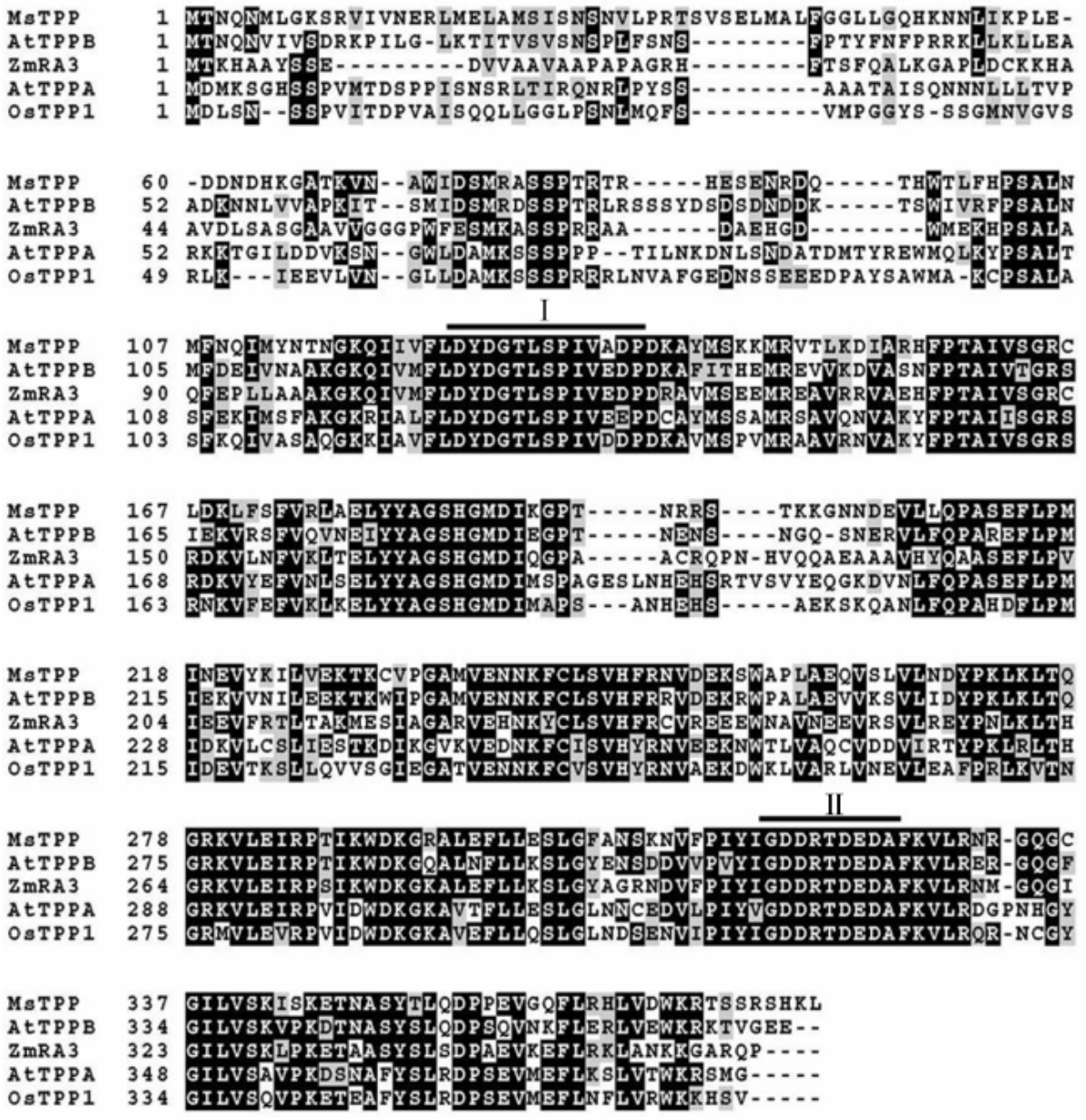

Fig. 1. Alignment of the deduced amino acid sequence of MsTPP with AtTPPA and AtTPPB from Arabidopsis, ZmRA3 from maize, and OsTPP1 from rice. Residues highlighted in black are identical in at least three protein sequences. The bars (BOX I and BOX II) represent highly conserved 'phosphatase boxes'. The accession numbers of TPPs are listed. Alfalfa: MsTPP(HM156211); Arabidopsis: AtTPPA(AF007778), AtTPPB(AF007779); Maize: ZmRA3(DQ436920); Rice: OsTPP1(AB120515).

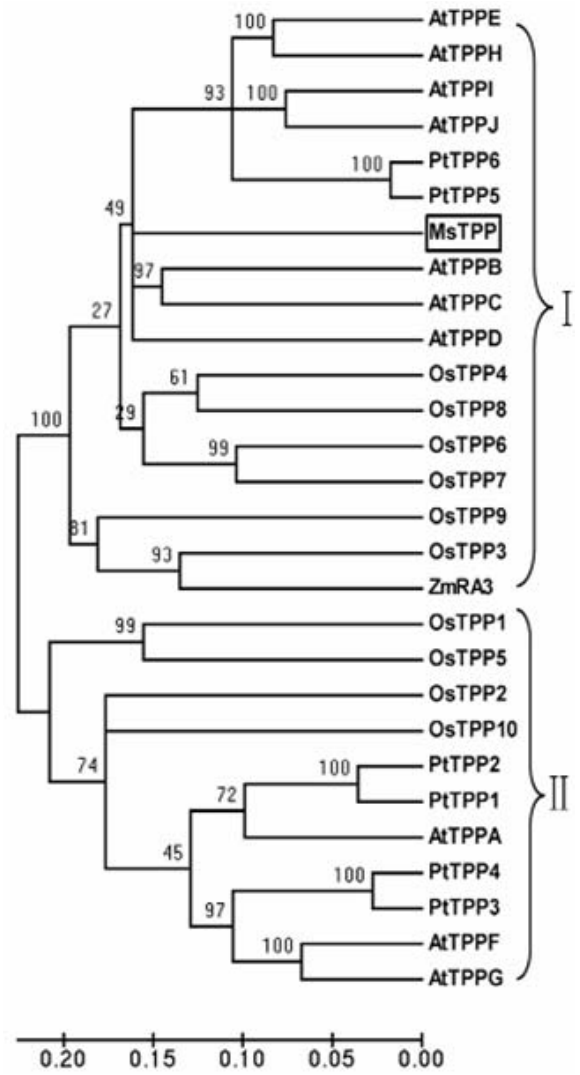

Fig. 2. A phylogenetic tree of MsTPP and other known TPP proteins in plants. MsTPP is indicated by a rectangle. Numbers at the branch points indicate bootstrapping values. A scale of distance is shown at the bottom.

Expression pattern of the *MsTPP*: The *TPP* family members encoded functional TPP enzymes and were known to influence plant growth and development through introducing trehalose biosynthesis (Eastmond *et al*., 2002; Avonce *et al*., 2006; Satoh-Nagasawa *et al*., 2006; Vandesteene *et al*., 2012). Semiquantitative RT-PCR analysis was carried out with different tissues of alfalfa shoot, including root, stem, leaf and petiole. As shown in Fig. 3A, *MsTPP* expression was tissue-specific in alfalfa seedling. *MsTPP* was strongly expressed in the root and weakly in petiole, and the transcript of *MsTPP* was barely detectable in stem and leaf. In *Arabidopsis*, *TPP* genes have also shown tissue-specific expression patterns, such as *TPPA* and *TPPB* with high expression in the cotyledons and different region of roots; whereas *TPPD* alone expressed more specifically in suspension cells (Li *et al*., 2008; Vandesteene *et al*., 2012).These results indicate that *TPPs* from different species might play different roles during plant development.

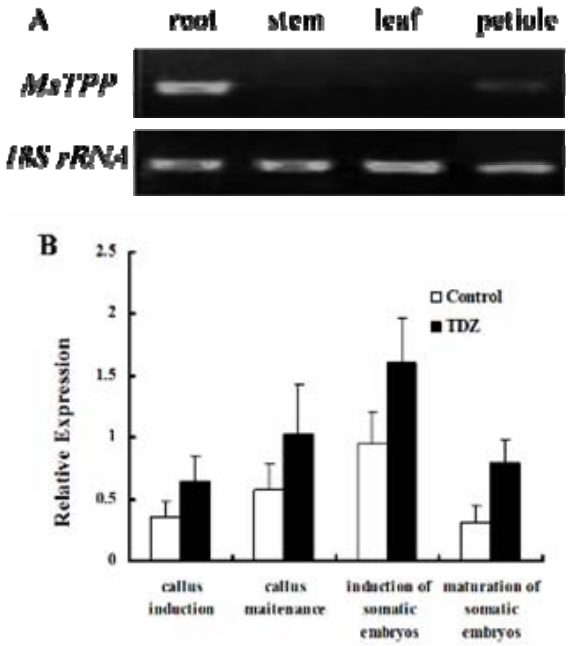

Fig. 3. *MsTPP* expression pattern in the tested tissues or cultures of alfalfa. (A) Semiquantitative RT-PCR analysis of transcript levels for *MsTPP* in different tissues. *18s rRNA* was used as an internal control. (B) *MsTPP* expression in response to TDZ treatment in cultures at different stage of somatic embryogenesis. Treatment concentration of TDZ was 0.93 μM, and the control was carried out as the cultures without TDZ treatment. Each value represents the means of three replicates, and *vertical bars* indicate the standard deviations (SD).

Thidiazuron (TDZ), a substituted phenylurea compound with high cytokinin-like activity, was often used to regulate morphogenesis in plant tissue culture (Mok *et al*., 1982; Mithila *et al*., 2003; Ma *et al*., 2011; Naz *et al*., 2011; Sivanesan *et al*., 2012). To analyze the effect of TDZ treatment on *MsTPP* expression during the whole period of somatic embryogenesis, the transcript level of *MsTPP* gene was examined by real-time PCR. As shown in Fig. 3B, TDZ treatment up-regulated the expression of *MsTPP* gene in cultures at each stage of somatic embryogenesis. In our previous study, it has been shown that TDZ completely suppressed the expression of embryogenic competence and *MsTPP* was specifically screened from the non-embryogenic callus of alfalfa (Zhang *et al*., 2006). Thus, these results indicate that *MsTPP* might be involved in the TDZ-induced morphogenesis in somatic embryogenesis of alfalfa.

Effect of TDZ on *MsTPP* expression in relation to embryogenic competence: The role of TDZ in morphogenesis is related to the concentration of TDZ adopted in the experiment (Mithila *et al*., 2003; Chhabra *et al*., 2008; Ma *et al*., 2011). To understand the effect of TDZ on the expression of *MsTPP* and the change of callus embyogenic competence, 0.93 and 50 μM TDZ were adopted in the stage of callus maintenance. The morphological changes in the callus after a 28 days culture were shown in Fig. 4A. The control callus appeared yellowish on the B5h medium, with the visible

somatic embryos developing on the surface callus (Fig. 4A-a). On the contrary, some green granules covered the surface of callus treated by 0.93 μM TDZ (Fig. 4A-b). When 50 μM TDZ was added into the B5h medium, those green structures reduced sharply on the callus surface (Fig. 4A-c). Following the stages of somatic embryo induction and maturation, the number of somatic embryos induced from these different calli was counted (Fig. 4B). The result revealed that the frequency of somatic embryogenesis in the calli decreased distinctly either 0.93 or 50 μM TDZ was adopted, indicating that embryogenic competence of the TDZ-treated callus significantly declined. Moreover, real-time PCR results showed that treatment with 0.93 and 50 μM TDZ at 28 days caused 2.44-fold and 1.55-fold level increase of *MsTPP* expression in the callus respectively, compared to that in control callus (Fig. 4C).

TDZ induced shoot organogenesis at concentrations lower than 2.5 μM, whereas at higher concentration (5-10 μM) somatic embryos were formed in African violent (Mithila *et al*., 2003). In lentil, TDZ at concentration lower than 2.0 μM induced shoot organogenesis whereas higher concentration (2.5-15 μM) caused a shift in regeneration from shoot organogenesis to somatic embryogenesis (Chhabra *et al*., 2008). Both somatic embryogenesis and adventitious shoot formation were induced from leaf and shoot explants with a higher concentration of TDZ (10-15 μM) whereas only adventitious shoots could be induced with low concentration of TDZ (5.0 μM) in *Ochna integerrima* (Ma *et al*., 2011). In our previous work, TDZ treatment could result in the loss of embryogenic competence of callus from alfalfa petiole and induce *MsTPP* expression in non-embryogenic callus (Huang *et al*., 1994; Zhang *et al*., 2006). In this study, although the morphogenesis of 50 μM TDZ-treated callus was highly similar with control callus and extremely differed with 0.93 μM TDZ-treated callus, the frequency of somatic embryogenesis from these TDZ-treated callus declined and the level of *MsTPP* expression increased in compared to the control. Dudits *et al*., (1991) have proposed that somatic embryogenesis is an extreme stress response of cultured plant cells *In vitro* and the embryogenic competence of explants cells might be subject to stress factors (Karami & Saidi, 2010). In somatic embryogenesis of some species, TDZ functions as a stress chemical and TDZ-induced somatic embryogenesis is regarded as stress-related response (Gairi & Rashid, 2004; Chhabra *et al*., 2008). However, our work revealed that the effect of TDZ on inhibition of embyogenic competence could be associated with up-regulation of stress-related *TPP* gene in somatic embryogenesis of alfalfa. Thus, it is interesting to note that obtaining of embryogenesis competence in somatic embryogenesis of alfalfa is associated with a stress-related *TPP* gene, but not depending on the stress condition with TDZ treatment. Further studies are needed to investigate whether *MsTPP* expression induced by TDZ is responsible for its depression effect on somatic embryogenesis of alfalfa by transgenic research.

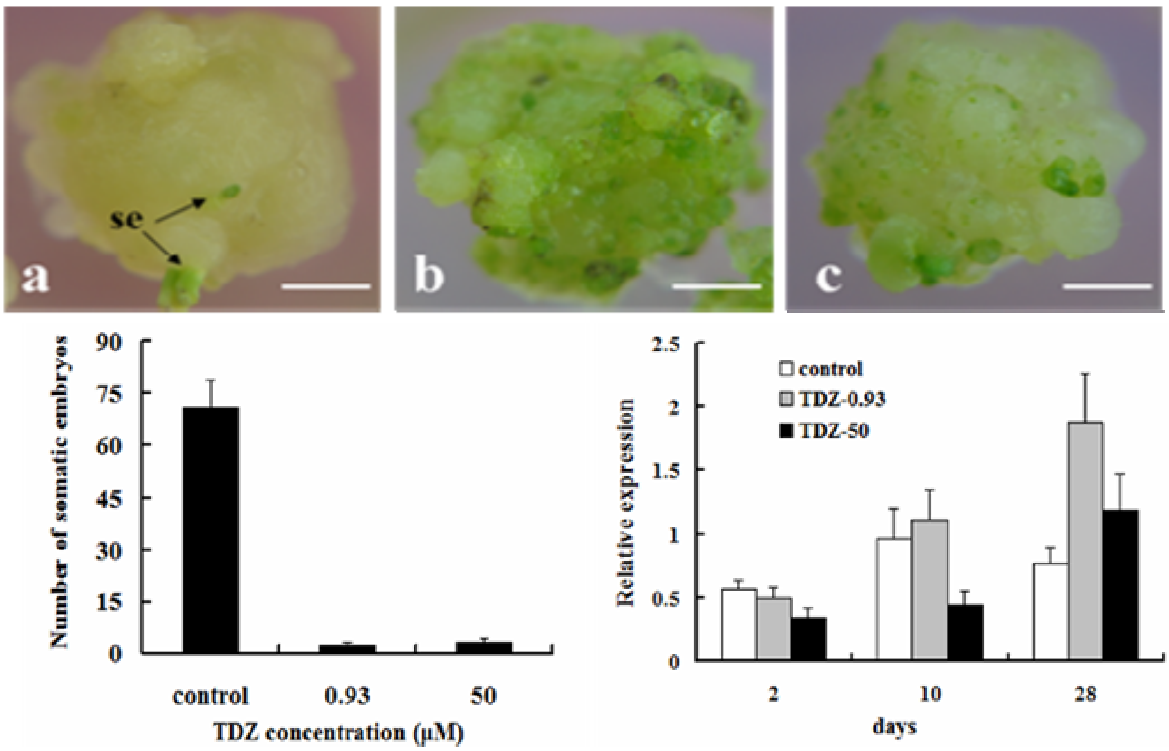

Fig. 4. Effects of 0.93 and 50μM TDZ on embryogenic competence and *MsTPP* expression in alfalfa callus. (A) Morphogenesis of alfalfa callus on control medium (a), TDZ-0.93 medium (b) and TDZ-50 medium (c). se, somatic embryo. Bar=3mm. (B) Number of somatic embryos developed from the control callus and TDZ-treated callus (after 14-day suspension culture in SH medium. n=3). (C) Relative expression level of *MsTPP* during callus maintenance, callus was cultured on control medium and 0.93 or 50 μM TDZ-treated medium for 2, 10 and 28 days, respectively. Each value represents the means of three replicates, and *vertical bars* indicate the standard deviations (SD).

Acknowledgments

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