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Molecular cloning and characterization of a cDNA encoding kiwifruit *L-myo-inositol-1-phosphate synthase*, a key gene of inositol formation

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Abstract L-*myo*-inositol-1-phosphate synthase (MIPS; EC 5.5.1.4) is the key enzyme involved in de novo synthesis of *myo*-inositol, leading to numerous cellular functions. We isolated an open reading frame of *Actinidia deliciosa MIPS* (*AdMIPS*), which is 1,533 bp long and codes for 510 amino acids, with a predicted molecular weight of 56.3 kDa. Sequence analysis revealed its high similarity with MIPS proteins from other organisms. Gene expression and enzyme activity were highest in flower and young fruit. Transcription of *AdMIPS* was also detected in other tissues. Moderate drought drastically induced expression in the leaves whereas salinity stress induced transcription and enzyme activity in the leaves, phloem, and roots with different degrees. However, a longer period of saline exposure suppressed both expression and enzyme activity in all sampled tissues, indicating that *AdMIPS* is salt-sensitive.

Keywords *Actinidia deliciosa* · Developmental stages · Drought · Enzyme activity · MIPS · Salinity

Introduction

L-*myo*-inositol-1-phosphate synthase (MIPS; EC 5.5.1.4) catalyzes the de novo synthesis of *myo*-inositol, which is highly conserved in both eukaryotes and bacteria. This is a two-step process. MIPS catalyzes the reaction from D-glucose-6-phosphate to L-*myo*-inositol-1-phosphate, which is then de-phosphorylated by inositol monophosphatase to release free *myo*-inositol [1, 2]. The former reaction is regarded as the committed step, making MIPS a rate-limiting enzyme. As the most abundant of the eight isomers of inositols, *myo*-inositol plays central roles in eukaryotic organisms [3]. Its metabolism, as well as that of derivatives, e.g., inositol phospholipids and inositol hexaphosphate, have vital functions in signal transduction, phosphate storage, membrane formation, stress tolerance, and the synthesis of metabolites such as ascorbic acid [4–7].

Gene cloning and characterization of *MIPS* have been conducted in animals, plants, yeast, bacteria, and green algae [8, 9]. In plants, the MIPS gene is part of a family in which different members seem to have divergent roles in embryo formation and seed growth [5, 10]. Numerous studies have concentrated on the relationship between plant MIPS and abiotic stress. Smart and Fleming [11] have reported that the transcript levels of *MIPS* in *Spirodela polyrrhiza* are induced by abscisic acid (ABA), while Chun et al. [12] have shown that MIPS transcription is down-regulated by salinity during sesame seed germination. Abreu and Aragão [8] have found that transcript levels in yellow passion fruit are differentially regulated by cold and heat and also respond to light stimulus, while research with *Jatropha curcas* has demonstrated the up-regulation of *MIPS* transcription and enzyme activity by ABA, drought, and NaCl treatments [13]. A salt-tolerant *MIPS* gene has been introduced into three genetically different species; transgenic plants exhibit increased inositol production and elevated salt tolerance under salinity stress [14]. Additionally, methylated inositols, such as D-ononitol and D-pinitol, have been associated with tolerances to drought and salinity [15, 16].

Kiwifruit (*Actinidia* sp.) is a commercially important fruit tree and breeding material all over the world, especially in China and New Zealand. One of its primary

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carbohydrates is *myo*-inositol, which accounts for 20–60 % of its total soluble sugar [17]. Drought and salinity are among the environmental stresses that plants most frequently encounter [18]. In some regions of the world, water deficit could be a severe challenge to fruit expansion of kiwifruit trees [19]. It is also known that kiwifruit seedlings accumulate *myo*-inositol under salinity stress [20]. Because little information is available about the activity of MIPS in that crop, we designed our experiment to focus on the molecular and enzymatic dynamics of MIPS during plant development and in response to stress.

Materials and methods

Plant materials and stress treatments

During the 2011 growing season (13 May to 28 September), fruit samples were collected at 15 day intervals, from 8-year-old vines of kiwifruit (*Actinidia deliciosa* cv. Qin Mei) at the horticultural experimental field of Northwest Agriculture & Forestry University, Yangling, China. At the mature fruit stage (28 September), shoot tips, phloem, petioles, carpopodia, and young and mature extended leaves were also collected. These samples were used for developmental and tissue-specific analyses of *AdMIPS*.

In a separate experiment, two-year-old kiwifruit plants were cultivated in individual plastic pots that were of approximately equal weight when the plants were added. Pre-treatment conditions for managing these seedlings followed those previously applied by our laboratory [21]. Drought was induced by withholding irrigation between 12 and 18 August, and before the treatment the maximum soil water capacity was calculated by stoving. Thereafter all pots were weighed daily at 8:00–9:00 a.m. When the soil water capacity reached 55, 45, or 35 % (mild, moderate, or severe drought, respectively), leaf samples were collected. Those from seedlings that continued to receive normal irrigation were used as controls. Immediately after samples from the severe-stress treatment were collected, we re-started the irrigation regimen, and collected more leaf samples after 1 day of this re-watering phase.

Salinity treatments followed time and concentration gradients. Before those experiments began, the seedlings were transferred to a growth room under a 16 h photoperiod (160 μmol m^{-2} s), 65 % humidity, and a 25 °C/21 °C (day/night) cycle. For 20 day, all plants were irrigated with tap water every 5 day to maintain a soil water capacity of 60–75 %. For the time gradient, 200 mM NaCl was added with the irrigation solution and leaves were collected at days 5, 10, 15, and 20. In addition, the effects of four NaCl concentrations were tested—100, 150, 200, and 250 mM. Each of these solutions was applied only once; thereafter tap water was supplied and any liquid that drained into containers below was poured back into each pot. As our control, some seedlings were irrigated with tap water alone. At the end of this 20 day period, leaf, phloem, and root samples (at least three for one replicate) were collected. All tissues were immediately frozen in liquid nitrogen and stored at −80 °C.

RNA extraction, cDNA synthesis, and cloning of MIPS

Total RNA was extracted from mature fruit by a modified cetyltrimethylammonium bromide (CTAB) method [22]. Prior to reverse-transcription, Rnase-free Dnase I (Invitrogen, USA) was added, per the manufacturer's instructions, to eliminate DNA contamination. First-strand cDNA was then synthesized with a RevertAid^{TM} First Strand cDNA Synthesis Kit (Fermentas).

The *AdMIPS* sequence was initially obtained through electronic assembly. *Arabidopsis MIPS* (GenBank Accession Number U04876) was used as a seed sequence that was aligned with Expression Sequence Tag (EST) sequences [23] of kiwifruit in NCBI. After each round of alignment, we chose five to eight EST sequences that were most similar to the seed sequence. They were assembled with DNAstar (http://www.dnastar.com/) and used as a new seed for more alignments until the acquired sequence could not be elongated further. A sense primer (5′-ATGTTTATCGAGAGC TTTAAG-3′) and antisense primer (5′-TCACTTGTACTC CAAAATC-3′) were designed according to this contig; their product contained the 1,533 bp open reading frame (ORF).

Thermocycling parameters included pre-denaturing at 94 °C for 8 min; then 38 cycles of 94 °C/45 s, 56 °C/45 s, and 72 °C/2 min; followed by a final step at 72 °C for 10 min. The amplified product (~1.5 kb) was purified from the agarose gel and ligased at 4 °C to the pGMET-Easy vector (Promega). The ligation mixture was used to transform Top10 competent cells and the transformants were selected on ampicillin plates. Single colonies with resistance were detected via PCR, and positive samples were sequenced with an ABI 3730 sequencer. The sequence data obtained for *AdMIPS* were checked against published MIPS sequences from other organisms and were analyzed with standard bioinformatics tools.

Phylogenetic analysis

AdMIPS and protein sequences of MIPS from 20 organisms were obtained from GenBank (www.ncbi.nih.nlm.gov) and aligned using Clustal W [24]. Phylogenetic analysis was conducted with MEGA (Molecular Evolutionary Genetic Analysis) version 5.0 software [25]. A neighbor-joining algorithm was used for constructing the

phylogenetic tree, and bootstrap values were computed with 1,000 replicates to evaluate support for the groupings.

Real-time PCR analysis of MIPS

RNA was extracted from plant tissues as described above. Reverse transcription was performed with 1 μg of RNA and a PrimeScript® RT reagent Kit (Takara). All of the reverse transcripts were adjusted with double-distilled water to a concentration of 150 ng uL^{-1}. A sense primer (5′-TCT CTCGGTCCCTCAAACTT-3′) and antisense primer (5′-CCCACATACGGCACATACTT-3′) were designed with Primer Premier, version 5.0, software (Palo Alto, CA). The RT-PCR was carried out by Bio-Rad iQ5 thermocycler in triplicate and the RT-PCR program included pre-denaturing at 94 °C for 3 min; then 45 cycles of denaturing at 94 °C for 30 s, annealing at 60 °C for 20 s, and elongation at 72 °C for 30 s. For the first cycle, we used a melt curve to ensure that the primers could not form dimers. Gene expression for each sample was normalized against house-keeping gene *Actin* with sense primer (5′-GCTTACAGAGGCACCACTCA ACC-3′) and antisense primer (5′-CCGGAATCCAGCA CAATACCAG-3′) and analyzed with Bio-Rad iQ5 optical system software.

Crude enzyme extraction and study of AdMIPS activity

Samples from the flowers, leaves, phloem, and roots were ground in a tenfold volume of extraction buffer (Tris–HCl; pH 7.5) containing 10 mM NH_4Cl, 10 mM β-mercaptoethanol, and 2 mM phenylmethylsulfonyl fluoride that was supplemented with 1 % Triton X-100 and 4 % polyvinylpolypyrrolidone. The homogenates were centrifuged for 30 min at 4 °C and 10,000 *g*. The supernatant was collected and stored at 4 °C, with one portion designated for detecting enzyme activity and the other for determining the soluble protein content. Enzyme activity was monitored according to the method of Barnett et al. [26] and was presented as nmol inositol-1-phosphate formed in each milligram of soluble protein within 1 h of reaction. The amount of soluble protein was measured as described by Bradford [27].

Results

Cloning and characterization of MIPS gene in *Actinidia deliciosa*

Through electronic assembly, we created a 1,756 bp contig and cloned a complete ORF sequence of MIPS from *Actinidia deliciosa*. This ORF contains 1,533 bp, coding for a protein with 510 predicted amino acids (Fig. 1). We have deposited this 1,533 bp sequence into GeneBank under Accession Number: JX122766. A comparison with protein information from other organisms revealed its high similarity with enzyme in eukaryotes; alignment was more divergent with prokaryotes, as previously described [28, 29]. When aligned with other plant MIPS protein sequences, four stretches of 'highly conserved' amino acids were also found in the *AdMIPS* sequence—GWGGNNG, LWTANTER, NGSPQNTFVPGL, and SYNHLGNNDG (Fig. 2). This is presumed to be essential for substrate-binding to the MIPS enzyme, perhaps pointing toward a conserved 'core structure' for catalytic activity through evolution [28]. A phylogenetic tree for *MIPS* from different species produced clustering into four groups—plant, animal, alga, and bacterium (Fig. 3). Terrestrial and aquatic plants had clearly been segregated into two groups early whereas the divergence of dicotyledons and monocotyledons within terrestrial plants

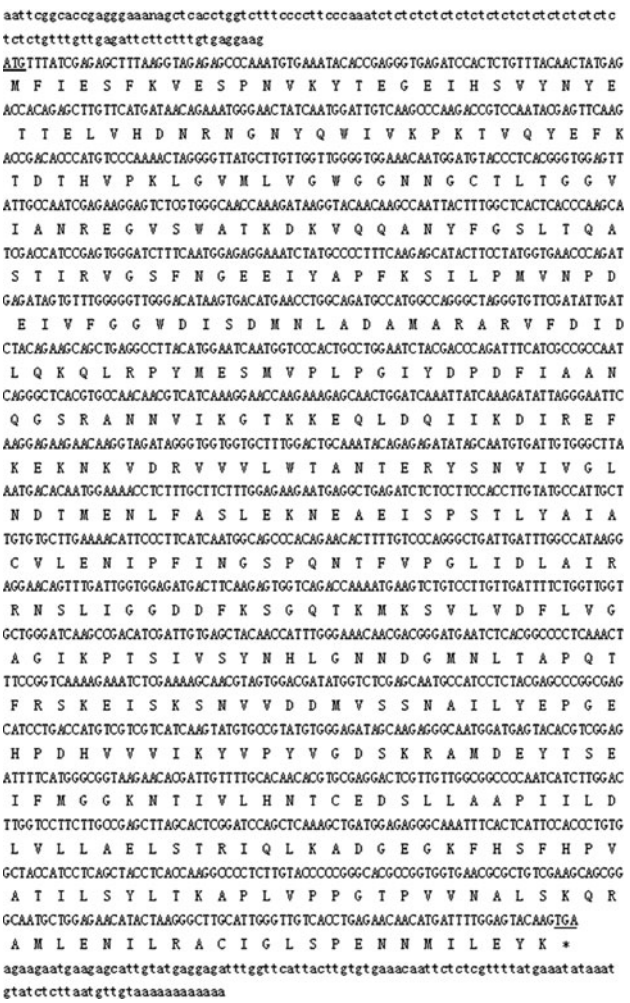

Fig. 1 *AdMIPS* cDNA and deduced amino acid sequence. *Uppercase letters* indicate sequence of clone ORF. Initiation and termination codons are *underlined*

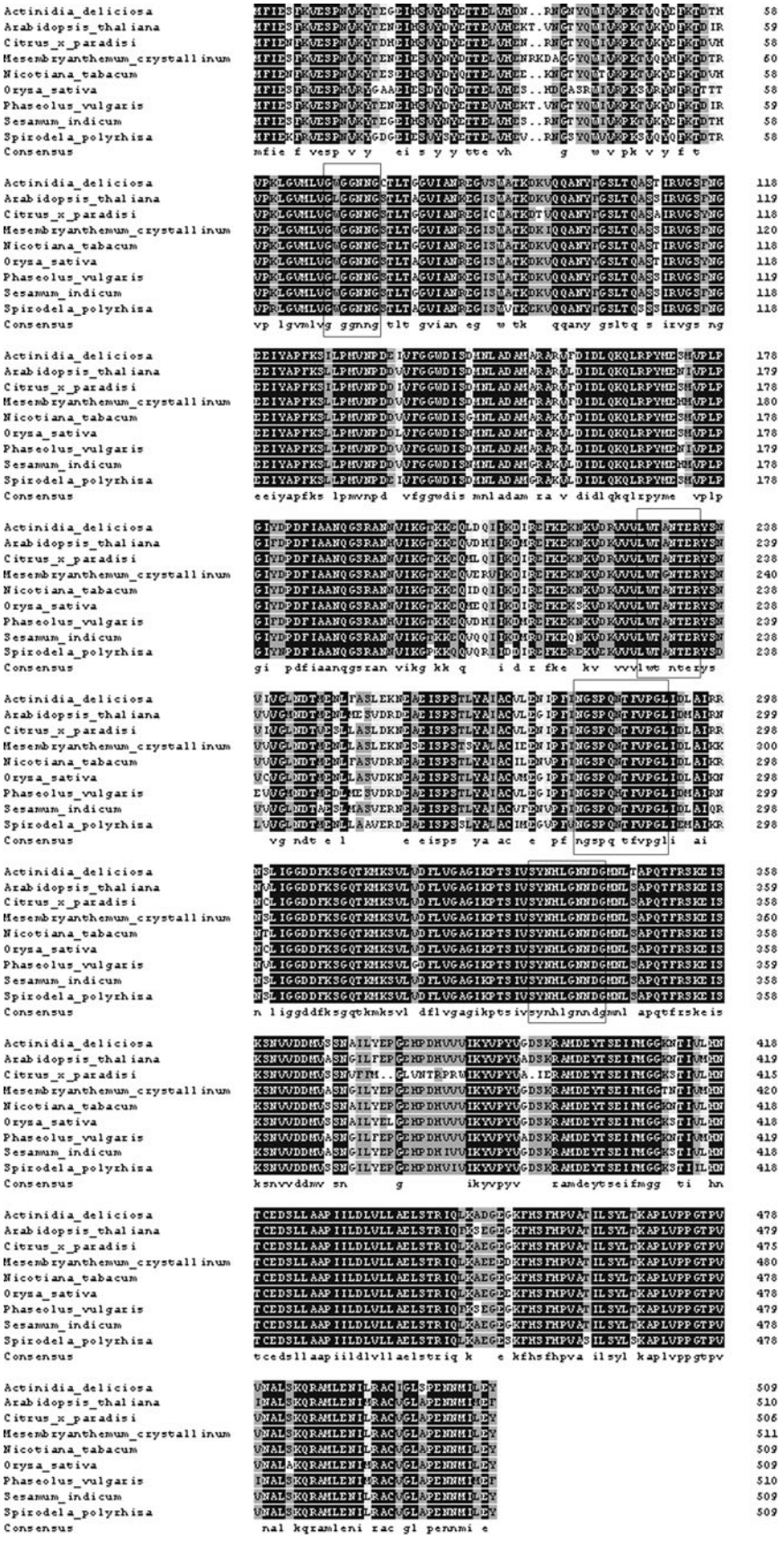

Fig. 2 Alignment of MIPS amino acid sequences from different plants. *Boxed residues* indicate four conserved stretches. GenBank Accession Numbers for nucleotides include: *Arabidopsis thaliana*, U04876; *Sesamum indicum*, AF284065; *Phaseolus vulgaris*, U38920; *Nicotiana tabacum*, AB009881; *Citrus paradisi*, Z32632; *Actinidia deliciosa*, JX122766; *Mesembryanthemum crystallinum*, U32511; *Oryza sativa*, AB012107; *Spirodela polyrhiza*, Z11693

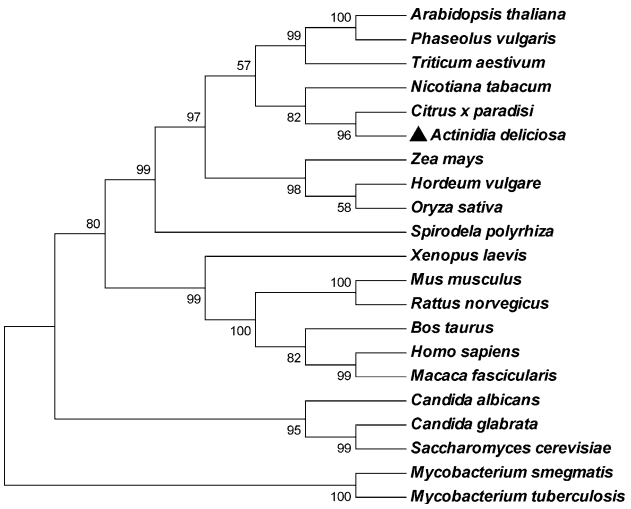

Fig. 3 Phylogenetic analysis of MIPS proteins from different organisms. *AdMIPS* in phylogenetic tree is marked by *triangle*. GenBank Accession Numbers for nucleotides include: *Arabidopsis thaliana*, U04876; *Phaseolus vulgaris*, U38920; *Triticum aestivum*, AF120146; *Nicotiana tabacum*, AB009881; *Citrus paradisi*, Z32632; *Actinidia deliciosa*, JX122766; *Mesembryanthemum crystallinum*, U32511; *Sesamum indicum*, AF284065; *Zea mays*, AF056326; *Hordeum vulgare*, AF056325; *Oryza sativa*, AB012107; *Spirodela polyrhiza*, Z11693; *Xenopus laevis*, BC077437; *Mus musculus*, AF288525; *Rattus norvegicus*, AABR03100304; *Bos taurus*, BC111160; *Homo sapiens*, AF207640; *Macaca fascicularis*, AB168239; *Candida albicans*, L22737; *Candida glabrata*, CR380955; *Saccharomyces cerevisiae*, L23520; *Mycobacterium smegmatis*, CP000480; *Mycobacterium tuberculosis*, BX842572

occurred later in the evolutionary process. Interestingly, *Triticum aestivum* clustered with dicotyledons rather than with other monocotyledons such as *Zea mays*. *AdMIPS* was most closely related to that from *Citrus paradisi*, a tropical fruit.

MIPS gene expression and enzyme activity of *A. deliciosa* fruits at different developmental stages

Transcript levels for *AdMIPS* were highest at days 0 and 15 after flowering, being three to four times greater than at day 30. Thereafter, expression declined gradually, almost to zero (Fig. 4a).

Changes in enzyme activity followed a trend similar to that for gene expression, with activities on days 0 and 15 being five to six times higher than measured on day 30. Afterward, activities remained relatively stable, increasing only slightly to just less than 2 nmol h^{-1} mg^{-1} at day 105 before dropping to the level recorded at day 30 (Fig. 4b).

Expression of *AdMIPS* in different tissues of kiwifruit

AdMIPS transcription was detected in all six tissue types. Compared with levels found for the house-keeping gene *actin*, expression of *AdMIPS* was higher in the petiole than

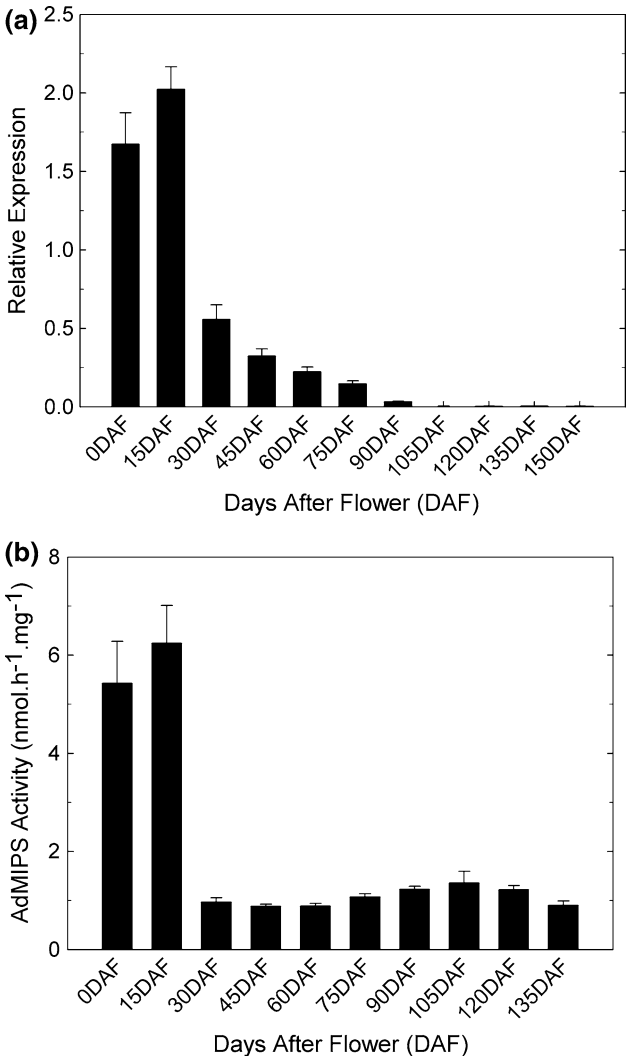

Fig. 4 Expression of *AdMIPS* (**a**) and enzyme activity (**b**) in fruits at different developmental stages (expression is shown as relative fold of mRNA level normalized to *actin*)

in any other tissue. Whereas shoot tips and mature leaves showed relatively higher expression, much less was detected in the young leaves, carpopodium, and phloem (Fig. 5).

Expression profile and enzyme activity of MIPS in *A. deliciosa* plants subjected to drought or salinity

Relative expression by *AdMIPS* was about 3 times (mildly drought-stressed plants) and 25 times higher (moderately drought-stressed plants) than the control respectively. Under severe drought, the transcription level decreased by approximately two-thirds of the highest amount, then, after 1 day of re-watering, declined to the level measured in the control (Fig. 6a). Enzyme activity of MIPS did not follow a similar trend but, instead, showed no significant difference among stress severities. After 1 day of re-watering, enzyme

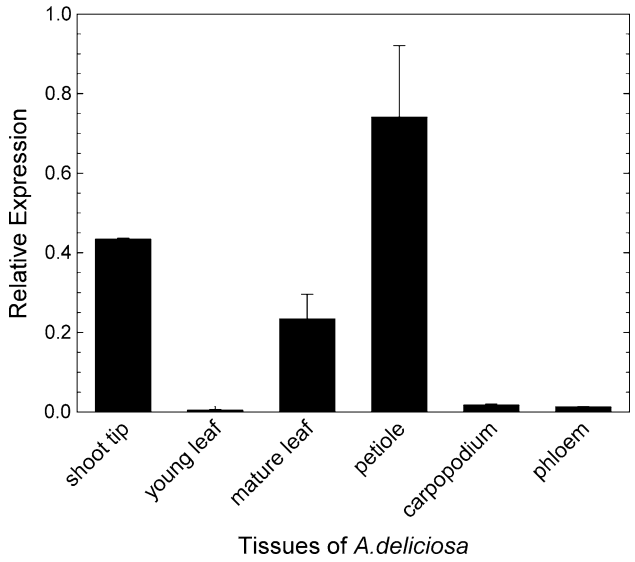

Fig. 5 Expression of *AdMIPS* in various tissues (shown as relative fold of mRNA level normalized to *actin*)

activity continued to decrease significantly to just above 2 nmol h^{-1} mg^{-1}. (Fig. 6b).

Under 200 mM NaCl treatment, transcription levels were 1.5 and 3.5 times higher than the control at days 5 and 10 after treatment, respectively. Afterward, expression declined sharply to one third of the control level at day 15 and to less than one-quarter of the control at day 20 (Fig. 6c). MIPS activity showed similar changes in response to salinity stress, rising gradually at days 5 and 10 before being repressed to levels below those of the control at days 15 and 20 (Fig. 6d).

Under control conditions, *AdMIPS* expression was identical between leaves and phloem. However, the expression in leaves was induced by 150 mM NaCl, repressed by 100, 200 and 250 mM NaCl; whereas that in phloem was decreased only by 250 mM NaCl. Root tissues from control plants showed the lowest expression; all four tested concentrations of NaCl led to an increase in *AdMIPS* transcription there (Fig. 6e). For the MIPS activity of these tissues, a converse tendency in leaves and roots was observed. Except at 150 mM NaCl, enzyme activity in the leaves was increased at all other concentrations whereas the highest level of enzyme activity in the roots was found in the control. The AdMIPS activity was slightly induced in the phloem by 100 mM NaCl (Fig. 6f).

Discussion

AdMIPS is a highly conserved enzyme

Previous studies of the nucleotide sequences and protein structures of *myo*-inositol-1-phosphate synthase have provided supporting evidence that MIPS is a highly conserved enzyme throughout the eukaryotic and prokaryotic phyla [28]. Although those groups tend to segregate into two divergent branches of the phylogenetic tree, they show high similarity in amino acid sequences. The 'core catalytic domains' present in both groups suggest two critical functions. First, MIPS catalyzes the de novo synthesis of *myo*-inositol, which is a precursor to several important metabolites, e.g., phytic acid, raffinose, ascorbic acid, and phosphoinositides. Second, it can act directly as an osmoprotectant for cellular functions [3].

Young fruit and reproductive tissue are major sources of AdMIPS

Many research groups have examined the relationship between seeds and MIPS because of the abundance of inositol hexaphosphate (phytic acid), the storage form of phosphate [30]. MIPS genes have been shown to be differentially expressed in *Arabidopsis* embryos at different developmental stages [5]. However, little information is available about MIPS expression in fruit trees. We detected MIPS gene expression and enzyme activity at several developmental stages in kiwifruit, especially the young fruit and flowers, which are major sources of *AdMIPS*. These findings are corroborated by previous reports [17, 31, 32]. This high amount of *myo*-inositol in young fruit may be associated with the maintenance of turgor during the cell expansion phase [31]. Additionally, high detection in the flowers might indicate a reproductive role for MIPS in kiwifruit. At the mature fruit stage, transcripts of *AdMIPS* were found in all tissue types that we collected, suggesting that MIPS expression is ubiquitous. Thus, the coordination of inositol metabolism and cellular growth can be achieved through such differential regulation [1, 33]. Several species, such as *Zea mays* [34], *Glycine max* [35], and *Arabidopsis thaliana* [36] contain more than one MIPS, and various isoforms have been localized to different organs in *Arabidopsis*, also indicating tissue-specific roles [5].

AdMIPS is regulated by drought or salt stress

MIPS can be induced upon exposure to drought, salinity, freezing, darkness, or treatment with ABA, demonstrating its important function in response to environmental stresses [8, 12, 37]. Here, under drought or a high NaCl concentration, gene expression and enzyme activity of *AdMIPS* were induced differentially. Despite a transient increase, both then declined gradually, indicating that *AdMIPS* could be suppressed at both the transcript and enzyme levels as the stress period lengthened. MIPS from a salt-tolerant rice has shown enhanced mRNA expression and retained a

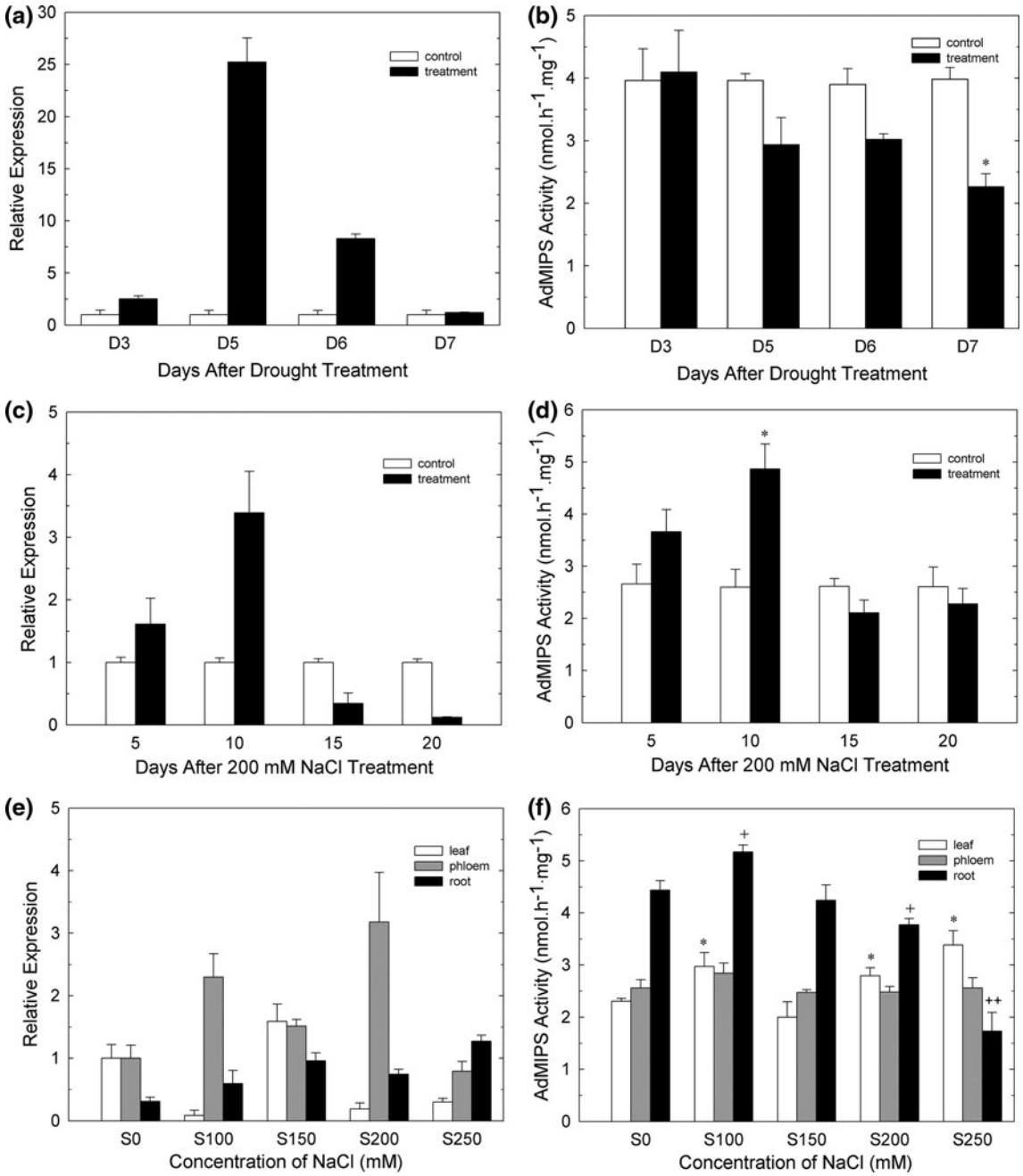

Fig. 6 **a** Expression of *AdMIPS* in leaves under increasingly severe drought stress and re-watering. **b** Enzyme activity of *AdMIPS* in leaves during drought and re-watering treatments. For **a** and **b**, D3, D5, D6 represent drought treatment for 3, 5, and 6 days (mild, moderate, and severe levels) respectively. D7 is 1 day after re-watering. **c** Time courses for *AdMIPS* expression in leaves under 200 mM NaCl treatment. **d** Enzyme activity of AdMIPS in leaves under 200 mM NaCl stress. **e** Expression of *AdMIPS* in leaves, phloem, and roots after 20 day of treatment with various NaCl concentrations. **f** Enzyme activity of AdMIPS in leaves, phloem, and roots after 20 day of treatment with various NaCl concentrations. For **e** and **f**, S0, S100, S150, S200, S250 represent salt concentration of 0, 100, 150, 200 and 250 mM respectively. Significant differences from control values are indicated by * (for leaves) and $^{+}$ (for roots); * and $^{+}$, $P < 0.05$; ** and $^{++}$, $P < 0.01$. Data are mean ± SE of 3 independent extracts

constant level of enzyme activity even under high salt concentrations. By contrast, the transcription of *MIPS* is not induced under saline conditions in *Arabidopsis thaliana*, suggesting an essential difference in response between halophytes and glycophytes [15, 38]. *Myo*-inositol accumulation was detectable before kiwifruit seedlings showed any signs of salt stress [20]. Our experiment confirms this result at transcription and enzyme levels. *Actinidia deliciosa* is known not salt- or drought-tolerant. However, the increase in transcript, enzyme and *myo*-inositol levels

may still suggest some protection to plant. *Myo*-inositol may have functions such as osmotic protection, scavenging oxygen radicals or storage of unused carbohydrate under osmotic stress [3, 20]. In other plant species, *MIPS* transcription or its enzyme activity were also induced by salt, drought or heat stress within 24 h [8, 13], indicating the importance of *myo*-inositol at the early stage of stress. When exposed to salinity stress, the higher enzyme activity in roots versus leaves/phloem might suggest that green tissues are rapidly impaired while roots may continue to elongate as they take up water from deeper soil layers [18].

To summarize, we have cloned a complete ORF of MIPS from *A. deliciosa*, and analyzed its transcription and enzyme activity in terms of fruit development, tissue specificity, and responsiveness to abiotic stresses. *AdMIPS* is an evolutionarily conserved enzyme, showing high similarity with *MIPS* from other plant sources. It might serve as a key element in young fruit given its high expression and level of enzyme activity. As reported with other glycophytes, *AdMIPS* can be induced by drought or salinity at both the mRNA and enzyme levels [8, 12]. Engineering of enzymes that catalyze the production of osmoprotectants is a useful tool for improving stress tolerance in plants [39]. In addition, *MIPS* tends to present as multigene families in plants, with each member appearing to be tissue-specific and having different functions [5]. To gain greater insight into the mechanism of *myo*-inositol metabolism in kiwifruit, further research should focus on the overexpression and downregulation of *AdMIPS* gene(s).

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