Planta (2013) 237:321–335
DOI 10.1007/s00425-012-1781-0

ORIGINAL ARTICLE

Ectopic expression of the ABA-inducible dehydration-responsive chickpea L-*myo*-inositol 1-phosphate synthase 2 (*CaMIPS2*) in Arabidopsis enhances tolerance to salinity and dehydration stress

Harmeet Kaur · Pooja Verma · Bhanu Prakash Petla · Venkateswara Rao · Saurabh C. Saxena · Manoj Majee

Received: 7 July 2012 / Accepted: 2 October 2012 / Published online: 13 October 2012

Abstract *Myo*-inositol participates in many different aspects of plant physiology and *myo*-inositol 1-phosphate synthase (MIPS; EC 5.5.1.4) catalyzes the rate limiting step of inositol biosynthetic pathway. Chickpea (*Cicer arietinum*), a drought-tolerant leguminous crop plant, is known to accumulate increased inositol during dehydration stress. Previously, we reported two differentially expressed divergent genes (*CaMIPS1* and *CaMIPS2*) encoding two MIPS isoforms in chickpea. In this communication, we demonstrated that *CaMIPS2* is an early dehydration-responsive gene and is also rapidly induced by exogenous ABA application, while *CaMIPS1* expression is not much influenced by dehydration or ABA. The regulation of expression of these two genes has been studied by examining their promoter activity through GUS reporter gene and differential promoter activity has been observed. Moreover, unlike *CaMIPS1* promoter, *CaMIPS2* promoter contains CRT/DRE *cis*-regulatory element which seems to play a key role in dehydration-induced expression of *CaMIPS2*. Furthermore, *CaMIPS1* and *CaMIPS2* have been successfully complemented and shown to repair the defect of seedling growth and altered seed phenotype of *Atmips1* mutant. Moreover, Arabidopsis transgenic plants overexpressing *CaMIPS1* or *CaMIPS2* exhibit improved tolerance to salinity and dehydration stresses and such tolerance of transgenic plants is correlated with their elevated level of inositol. Remarkably, *CaMIPS2* transgenic lines perform better in all attributes than *CaMIPS1* transformants under such stress conditions, due to comparatively unabated production of inositol by CaMIPS2 enzyme, as this enzyme retains significant activity under stress conditions.

Keywords Abiotic stresses · Functional complementation · Gene duplication · Promoter analysis · Subfunctionalization · Transgenic

Abbreviations

GUS	β-Glucuronidase
IMP	Inositol monophosphatase
MIPS	*Myo*-inositol 1-phosphate synthase
VC	Vector control

Introduction

Inositol, a six-carbon cyclo-hexane hexitol, is found ubiquitously across the biological kingdom and is an essential component of eukaryotic cell. The role of inositol in plants is manyfold, though inositol is primarily essential for plant growth and development. Inositol is a precursor for many inositol containing compounds and is implicated in various physiological and biochemical processes such as growth regulation, cell membrane biogenesis, hormonal regulation, stress signaling, etc. (Loewus and Murthy 2000; Stevenson et al. 2000). Recent studies suggest that inositol also plays a significant role in plant immunity and programmed cell death (Meng et al. 2009; Chaouch and Noctor 2010; Donahue et al. 2010). Apart from these, inositol and its few derivatives mainly pinitol have also been shown to be implicated in abiotic stress adaptation, possibly by protecting cellular structures from reactive oxygen species and by

Electronic supplementary material The online version of this article (doi:10.1007/s00425-012-1781-0) contains supplementary material, which is available to authorized users.

H. Kaur · P. Verma · B. P. Petla · V. Rao · S. C. Saxena · M. Majee (✉)
National Institute of Plant Genome Research,
Aruna Asaf Ali Marg, New Delhi 110067, India
e-mail: majeem@yahoo.co.uk; manoj_majee@nipgr.res.in

controlling turgor pressure (Paul and Cockburn 1989; Ishitani et al. 1996; Sheveleva et al. 1997; Nelson et al. 1998; Shen et al. 1999; Loewus and Murthy 2000). In addition, *myo*-inositol is utilized in abiotic stress-induced raffinose and galactinol synthesis (Taji et al. 2002). Inositol is biosynthesized via a two-step pathway which is highly conserved among all classes of organisms. In this pathway, *myo*-inositol 1-phosphate synthase (MIPS: EC 5.5.1.4) catalyzes the first and rate limiting step and specifically converts D-glucose 6-phosphate to L-*myo*-inositol 1-phosphate. *Myo*-inositol 1-phosphate is further dephosphorylated to produce free inositol by the enzyme inositol 1-phosphate phosphatase (IMP: EC 3.1.3.25) (Loewus and Loewus 1983; Loewus 1990). Free inositol can then be channelized to different pathways and produces various inositol derivatives including ononitol and pinitol. The rate limiting enzyme MIPS has been reported from various sources and MIPS-coding sequences have been cloned and characterized from widely different organisms including plants (Majumder et al. 2003). Regulation of *MIPS* gene expression has been studied in few plants and appears to be species specific (Yoshida et al. 1999; Hegeman et al. 2001; Chun et al. 2003; Abreu and Aragao 2007). In response to stresses, increased accumulation of pinitol or inositol as a consequence of transcriptional induction of the MIPS-coding gene has been reported in few plants like *Mesembryanthemum crystallinum, Cicer arietinum*, etc. (Ishitani et al. 1996; Boominathan et al. 2004). In addition, introgression of MIPS-coding gene from *Porteresia coarctata* (*PcINO1*) was shown to confer salt tolerance in evolutionary diverse organisms including plants like tobacco, rice and *Brassica juncea* (Majee et al. 2004; Das Chatterjee et al. 2006). Multiple MIPS-coding genes have been reported in some plant species like maize, soybean, Arabidopsis and their differential expression patterns were also shown in respective plant species (Yoshida et al. 1999; Abreu and Aragao 2007; Donahue et al. 2010). In *Arabidopsis thaliana*, three MIPS-coding genes (*AtMIPS1, 2, 3*) have been reported and each member has been shown to play a specific physiological function. Among these three MIPS-coding genes, *AtMIPS1* was shown to have a greater contribution to *myo*-inositol levels in Arabidopsis plant (Meng et al. 2009; Donahue et al. 2010; Luo et al. 2011). The presence of such multiple MIPS genes in plant species thus suggests their participation in specific physiological functions.

Chickpea is an annual self- pollinated crop plant and is mostly grown in the arid and semi-arid regions of the world. Chickpea is a major source of human food as rich source of protein particularly for developing countries. The productivity of this grain legume is usually low and further reduced by environmental stresses (Ahmad et al. 2005). Despite having an immense agronomic and nutritional importance, research in chickpea is very limited, probably due to the lack of efficient and dependable transformation protocol. In addition, non-availability of genome sequencing data and mutant resources further restrict the research in this grain legume. In this particular plant, inositol seems to play an important role in drought tolerance besides its role in growth and development, since inositol content and MIPS transcript were shown to be significantly increased under dehydration condition (Boominathan et al. 2004; Kaur et al. 2008). However, the molecular regulation of MIPS gene and its role in stress adaptation are largely unknown in this particular crop plant. Previously, we reported differentially expressed two divergent MIPS genes (*CaMIPS1* and *CaMIPS2*) from chickpea. Furthermore, CaMIPS2 enzyme has been shown to retain higher activity than CaMIPS1 in in vitro conditions at high temperature and salt concentration and provides better protection to transformed fission yeast under adverse conditions (Kaur et al. 2008).

In this study, we analyze dehydration-induced transcript accumulation of *CaMIPS2* in a time-course manner and the effect of ABA on its regulation. The differential regulation of *CaMIPS1* and *CaMIPS2* expression is investigated through promoter–GUS fusion analysis. To evaluate the potential role of these two genes in plant stress adaptation, we begin with the functional validation of these genes in Arabidopsis *MIPS1* mutant (*Atmips1*, SALK_023626). Finally, transgenic *Arabidopsis thaliana* Col-0 plants overexpressing *CaMIPS1* and *CaMIPS2* are generated and their growth responses are analyzed under various stress conditions.

Materials and methods

Plant material and treatments

Chickpea (*Cicer arietinum* L. cv BGD72) seeds were obtained from Indian Agricultural Research Institute (IARI), New Delhi, India. Seeds were germinated on wet germination paper. The germinated seedlings were transferred to soil in a 3:1 ratio of agropeat to vermiculite and grown at 20 ± 2 °C and 12 ± 2 °C at day and night temperatures, respectively, with 50 % relative humidity in growth room (Boominathan et al. 2004). Six-day-old seedlings were used for various stress and hormone treatments. Dehydration was imposed in a time-course manner starting from 15 min to 3 h as described previously (Boominathan et al. 2004). For hormone treatment, 6-day-old seedlings were treated with 100 µM ABA, IAA, SA or GA (Sigma, St. Louis, MO, USA) for 5 h according to method described by Syam Prakash and Jayabaskaran (2006).

Arabidopsis thaliana ecotype Columbia was used in this study and plants were grown in a 3:1 combination of agropeat and vermiculite, in growth room under controlled condition

(16/8 h light and dark cycle at 22 ± 2 °C, light intensity of 100 μmol photons m^{-2} s^{-1}) for general growth and seed harvesting unless otherwise mentioned.

RNA isolation and real-time PCR

Total RNA was isolated from the Arabidopsis or chickpea seedlings using TRI Reagent (Sigma). 2 μg RNA was treated with DNaseI (Ambion, Austin, TX, USA) and further used for cDNA preparation using ABI cDNA preparation kit (Applied Biosystems, Foster City, CA, USA). A 1:20 dilution of the cDNA was used for quantitative real-time PCR in a 20-μl reaction with 10 pM suitable primers as described by Kaur et al. (2008). Primers used in this study are listed in Supplementary Table S1.

Isolation of *CaMIPS1* and *CaMIPS2* promoter sequences

Promoter sequences were isolated by genome walking using genome walker kit (Clonetech, Carlsbad, CA, USA) following manufacturer's protocol. Genome walking PCR was performed on genomic DNA library using gene specific primers and the adaptor primers provided by the kit. Amplified fragments were then cloned into pGEMT easy vector (Promega, Madison, WI, USA) and sequenced. Primers used in this experiment are listed in Supplementary Table S1.

Arabidopsis thaliana transformation and transgenic analysis

The full-length *CaMIPS1* and *CaMIPS2* cDNAs (Accession no: EU714004 and EU715005) were cloned into plant expression vector pCAMBIA2301 (for generating overexpression lines) or pCAMBIA1301 (for mutant complementation) under the control of CaMV 35S promoter. Overexpression lines were made in *Arabidopsis thaliana* col-0 background. For mutant complementation, mutant line of Arabidopsis *MIPS1* (*Atmips1-2,* SALK_023626, Donahue et al. 2010) was used for transformation.

For promoter analysis, approximately 1 kb region upstream of the translational start site of *CaMIPS1* and *CaMIPS2* were fused independently to *β*-glucuronidase (GUS) reporter gene (Jefferson et al. 1987) in pKYLX 70 vector. A truncated *CaMIPS2* promoter: GUS construct was similarly generated, where CRT/DRE element of *CaMIPS2* promoter was deleted. Primers used to make these constructs are listed in Supplementary Table S1. Arabidopsis plants were transformed through *Agrobacterium*-mediated transformation via floral dipping method (Clough and Bent 1998). Transformed plants were selected by kanamycin or hygromycin resistance followed by PCR analysis.

Promoter analysis: *β*-glucuronidase (GUS) assay for histochemical expression pattern and quantification of GUS transcript

Independent and stably transformed homozygous promoter:GUS lines were stained for GUS activity. Seedlings, leaves, inflorescence, floral organs and siliques were detached and incubated overnight in GUS staining solution [100 mM phosphate buffer (pH 7.0), 1 mM X-gluc (Gold biotechnology, St. Louis, MO, USA), 10 mM EDTA, 0.1 % (v/v) Triton X-100] at 37 °C to check the tissue-specific activity of these promoters. Tissue samples were then treated with 95 % (v/v) ethanol to remove chlorophyll and photographed with microscope (Nikon stereo zoom). For quantification of GUS transcript under dehydration condition, seeds from T_3 homozygous transgenic lines of respective promoters: GUS were grown in 1/2 MS medium. Ten-day-old seedlings were subjected to dehydration by placing the seedlings on blotting sheet for 5 h under continuous low light conditions. A control set was maintained in the plates under same light conditions. Subsequently, RNA was isolated for the quantification of GUS transcript through real-time PCR.

Stress treatments for transgenic lines

For salt stress experiment at germination and seedling stage, homozygous seeds of wild type, vector control (designated as VC) and multiple independent overexpression lines of *CaMIPS1* and *CaMIPS2* were surface sterilized and grown in 1/2 MS0 media supplemented with 0, 100 and 200 mM NaCl. Germination was scored at 5 days. Seeds were considered to be germinated when the radicle protruded out from seed coat. Germinated seeds were further allowed to grow for 10 days and growth pattern was monitored. Mature plants were also assessed for salinity and dehydration stress tolerance. For salt stress, initially seedlings were grown in 1/2 MS0 media and then 14-day-old plants were transferred to pots and allowed them to grow for 14 more days. Finally, salt stress was imposed on these 4-week-old plants by watering them with 200 mM NaCl solution every alternate day for 12 days.

For dehydration stress, seedlings were similarly grown in 1/2 MS0 media and then 14-day-old plants were transferred to pots and allowed to grow for further 10 days with routine watering. Finally, dehydration stress was imposed on these 24-day-old plants by withholding water for 3 weeks. Further, the plants were allowed to recover under normal routine watering (Sunkar et al. 2003). After these treatments growth responses were monitored and various traits like plant height, weight, rosette diameter and silique number per plant were also measured. For oxidative stress treatment, leaves of overexpression lines and vector control

were floated in 10 μM of paraquat for 24 h in continuous white light to induce oxidative stress as described by Qian et al. (2007). Leaves floated in de-ionized water served as control for the experiment.

Chlorophyll estimation

Chlorophyll was extracted with 80 % (v/v) acetone according to the method of Arnon (1949). Absorption was measured spectrophotometrically at 663 and 645 nm and total chlorophyll content was calculated (Wellburn and Lichtenthaler 1984). The chlorophyll content was measured and normalized per gram of fresh weight sample.

Assay of MIPS enzyme

The enzyme was assayed colorimetrically by the periodate oxidation method of Barnett et al. (1970) with minor modifications as described in Kaur et al. (2008). The amounts of inorganic phosphate released from the MIPS reaction product upon periodate oxidation was estimated by the method described by Chen et al. (1956). Protein was estimated according to the method of Bradford (1976) using Bio-Rad protein estimation kit.

Estimation of inositol content

Inositol content of transgenic plants was estimated according to the method of Bieleski and Redgwell (1977) with minor modification. 10-day-old seedlings (~500 mg) were homogenized and total water-soluble sugars and polyols were extracted with a mixture of methanol–chloroform–water–trichloroacetic acid (12:5:2:1, by vol.). Subsequently, chloroform and water (1:1, v/v) was added for phase separation and the aqueous phase containing the bulk of the water-soluble plant metabolites including inositol was lyophilized.

For GC analysis, the lyophilized samples were transferred quantitatively to glass reacti-vials (Pierce Chemical Co.) by dissolving in water. Water was evaporated and samples were kept in a vacuum desiccator over P_2O_5 for complete removal of water. Samples were derivatized as described by Majee et al. (2004) and were run through gas liquid chromatography on a Chemito 1000 instrument by Chemito-Toshniwal, equipped with a flame ionization detector (FID). Quantifications were done by a computer using specific Clarity Lite software.

Statistical analysis

Student's *t* test was applied to the data. Differences between the transgenics and control plants were considered significant when $P < 0.05$.

Results

CaMIPS2 is an ABA-inducible early dehydration-responsive gene in chickpea

In our previous study, *CaMIPS1* and *CaMIPS2* genes were shown to be differentially expressed in organs and stressful environmental conditions (Kaur et al. 2008). Particularly under dehydration condition, *CaMIPS2* was found to be highly induced, while *CaMIPS1* expression remained unchanged or slightly induced. In this study, we further examined the transcript accumulation of *CaMIPS1* and *2* at various time points of dehydration stress in chickpea seedling through real-time PCR as described in "Materials and methods" section. Results revealed that after initial exposure of dehydration stress (15 min), a little difference was observed between *CaMIPS1* and *CaMIPS2* expression; however, after as early as half an hour of exposure, the differences of their expression became clearly apparent. After 1 h of dehydration stress, *CaMIPS2* transcript accumulation was found to be doubled than control, while *CaMIPS1* transcript remained unchanged (Fig. 1a).

Transcripts accumulation of these two genes was also investigated under various phytohormone treatments. To analyze this, chickpea seedlings were treated with various phytohormones and transcript accumulation was analyzed through real-time PCR. As shown in Fig. 1b, both genes appeared to be induced in presence of ABA; however, accumulation of *CaMIPS2* transcript was found to be significantly higher than *CaMIPS1* transcript in ABA-treated seedlings. Enhancement of transcript accumulation of *CaMIPS2* was also observed in salicylic acid-treated seedlings, while GA and IAA showed no significant effect on their expression (Fig. 1b). We further investigated the transcript accumulation of these two genes at various time points of ABA treatment in chickpea seedlings and *CaMIPS2* was found to be rapidly induced in as early as 15 min of ABA treatment. However, enhanced accumulation of *CaMIPS2* transcript was found to be progressively decreased in subsequent time points following ABA treatment (Fig. 1c).

CaMIPS1 and *CaMIPS2* promoters exhibit differential activity in organs and under dehydration stress

In our present and previous studies, *CaMIPS1* and *CaMIPS2* have shown to be differentially expressed in chickpea. To understand the molecular basis of their differential expression, the 5′ upstream regulatory regions (up to ~1 kb) of these two genes were cloned through genome walking PCR and further promoter analysis was carried out. For this purpose, constructs were made for each promoter in fusion with *β*-glucuronidase (*GUS*) reporter gene

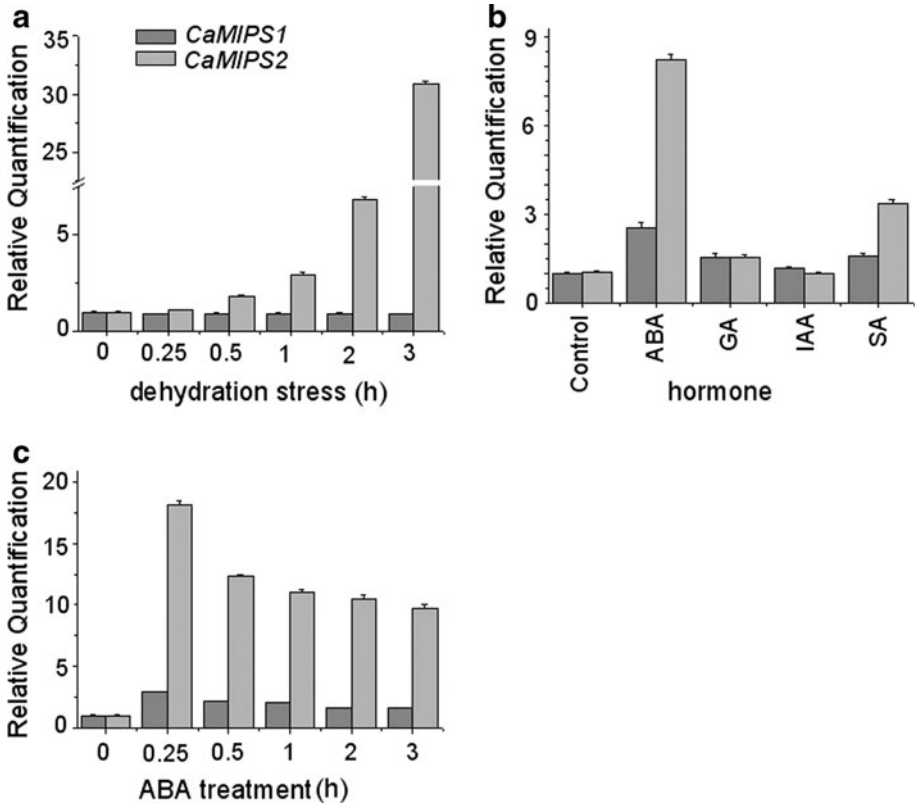

Fig. 1 Quantitative real-time PCR analysis of *CaMIPS1* and *CaMIPS2* transcript accumulation in chickpea seedling at different time interval of dehydration treatment (**a**), with different hormones treatment (**b**) and at different time interval of ABA treatment (**c**). Six-day-old seedlings were used for various stress and hormone treatments as described in "Materials and methods" section. In all experiments, 18SrRNA was used as an endogenous control and $\Delta\Delta C_T$ method (ABI) was used for relative quantification. Values are the result of triplicate analysis of three biological replicates. *Error bars* indicate the standard devianstion

(Jefferson et al. 1987) and introduced into *Arabidopsis thaliana.* Multiple T_3 transgenic Arabidopsis plants homozygous for *GUS* gene were used for further analysis. To check the promoter activity, intact seedlings and different tissues from various developmental stages from multiple independent lines were stained for GUS expression. *CaMIPS1* and *CaMIPS2* promoter-driven GUS expression was observed in all organs albeit at different levels, whereas in mature silique only *CaMIPS2* promoter-driven GUS expression was found (Fig. 2a). In seedling stage, *CaMIPS2* promoter-driven GUS expression was observed in almost all cells throughout the leaf, while *CaMIPS1* promoter-driven GUS expression was mostly restricted to veins or vascular tissues of leaf (Supplemental Fig. S2).

These results were found to be fairly similar with transcript analysis of these two genes in our previous study where *CaMIPS1* and *CaMIPS2* transcripts were observed in leaf, root, shoot and flower, but in seed only *CaMIPS2* transcript was detected (Kaur et al. 2008). Next, we investigated the regulatory mechanism of the differential expressions of *CaMIPS1* and *CaMIPS2* under dehydration stress. Towards this aim, both the promoters were examined for *cis*-regulatory elements for stress-induced expression through in silico analysis using PLACE and PlantCARE database (Higo et al. 1999; Lescot et al. 2002). Analysis revealed that both the promoters contain many similar putative *cis*-regulatory elements for ABA or stress-induced expression in different positions; however, few specific *cis*-regulatory elements responsible for dehydration-induced expression were noticed only in *CaMIPS2* promoter (Table 1). As for example, both the promoters were shown to contain ABRE element or MYC or MYB1 *cis*-regulatory elements, but CRT/DRE *cis*-regulatory elements were specifically found in *CaMIPS2* promoter. To examine whether these differences in their promoter regions are responsible for such differential expression of these two genes under dehydration stress, the quantitative *GUS* transcript analysis was done in respective transformed lines under normal and dehydration stress condition through real-time PCR. Under dehydration stress, all independent lines of *CaMIPS2* promoter-*GUS* transgenics were observed to accumulate 10–15 times more *GUS* transcript than normal condition, while *CaMIPS1* promoter-*GUS* transformed plants had fairly similar or slightly enhanced transcript accumulation in such dehydration condition. These results indicate that differences in their promoter activity might be responsible for differential transcript accumulation of these two genes under dehydration stress condition and the possible involvement of CRT/DRE element in dehydration-induced expression of *CaMIPS2*, as CRT/DRE element was previously shown to be involved in cold or dehydration-induced gene expression in many plant species (Yamaguchi- Shinozaki and Shinozaki 1994). Hence, we were interested in analyzing the *CaMIPS2* promoter without CRT/DRE

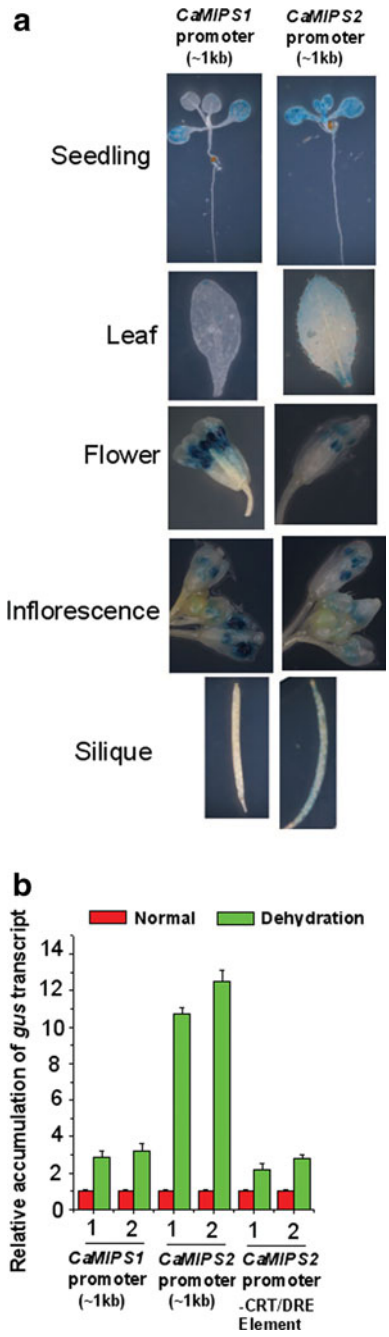

Fig. 2 Promoter analysis of *CaMIPS1* and *CaMIPS2* gene. The promoters from *CaMIPS1* and *CaMIPS2* were used to drive GUS expression in Arabidopsis plant. **a** Histochemical localization of GUS activity in seedlings, vegetative and reproductive organs of transformed Arabidopsis *CaMIPS1* and *CaMIPS2* promoter–GUS fusion lines. Only one representative line of each transgenic is shown here. **b** Quantitative transcript analysis of GUS reporter gene of *CaMIPS1*promo (1 kb):GUS, *CaMIPS2*promo(1 kb):GUS, *CaMIPS2* promo-CRT/DRE element: GUS transformed seedlings under normal and dehydration stress. Analyses of only two representative lines (*1* line 1 and *2* line 2) of each transgenic exhibiting GUS expression are shown here. Relative quantification was determined through real-time PCR. 18SrRNA was used as an endogenous control. *Error bars* indicate the SD of the mean

element (ATCGAC). Construct was similarly made with *CaMIPS2* promoter lacking CRT/DRE element and eventually *GUS* transcript accumulation was analyzed in transgenic plants under normal and dehydration stress condition. Results showed no significant induction of *GUS* reporter gene in dehydration condition when CRT/DRE element was absent in *CaMIPS2* promoter, as GUS transcript accumulation was found to be only slightly increased in dehydration stress condition compared to normal condition (Fig. 2b). This result clearly demonstrated that CRT/DRE elements in *CaMIPS2* promoter play a key role in dehydration-induced transcriptional activation of *CaMIPS2* gene. All these results were evaluated in multiple independent transgenic lines to rule out the possible insertional position effect factor of *GUS* gene.

CaMIPS1 and *CaMIPS2* functionally complement altered growth and development of Arabidopsis *Atmips1* mutant

Previously, lack of *MIPS1* in Arabidopsis mutant (*Atmips1-2 SALK_023626*) was shown to have altered growth and development including reduced seedling growth and wrinkled seed phenotype (Donahue et al. 2010). In order to check the ability of *CaMIPS1* and *2* in repairing such defects of *Atmips1-2* mutant, both *CaMIPS1* and *CaMIPS2* were ectopically expressed in *Atmips1-2* mutant under the control of ubiquitous 35S cauliflower mosaic virus promoter. Subsequently, we compared the growth pattern and seed phenotype of two representative independent lines expressing either *CaMIPS1* or *CaMIPS2* with empty vector-transformed or wild-type plant. As shown in Fig. 3, *Atmips1-2* mutant produced wrinkled seed and also displayed reduced growth at seedling stage particularly short root phenotype as compared to the wild-type plant as reported previously (Donahue et al. 2010). However, such plants when transformed with either *CaMIPS1* or *CaMIPS2* exhibited normal root and seed phenotype like wild type (Fig. 3). On the contrary, only vector without MIPS could not repair such defects of the mutant plant. This result clearly demonstrated that the expression of both *CaMIPS1* and *CaMIPS2* can repair the defect of root growth and seed phenotype caused by the absence of AtMIPS1 in such mutant.

CaMIPS2-transformed Arabidopsis plants exhibit enhanced salt and drought tolerance

In our previous experiment, we observed both *CaMIPS1* and *CaMIPS2* genes are functional in *Arabidopsis thaliana*. Here, we wanted to investigate the possible role of *CaMIPS1* and *CaMIPS2* in plant stress adaptation and whether *CaMIPS2* can impart better tolerance to the plant under

Table 1 List of putative *cis*-regulatory elements for ABA and stress-induced expression in chickpea *CaMIPS1* and *CaMIPS2* promoter regions

Promoter	*cis*-Regulatory element	Sequence	Start position	Reference
CaMIPS1	ABRELATERD1	ACGTG	−195	Nakashima et al. (2006)
	ACGTATERD1	ACGT	−195	Nakashima et al. (2006)
	CACGTGMOTIF	CACGTG	−196	Busk and Pages (1998)
	MYB1AT	WAACCA	−481	Abe et al. (2003)
	MYCCONSENSUSAT	CANNTG	−196	Abe et al. (2003)
CaMIPS2	ABRELATERD1	ACGTG	−139	Nakashima et al. (2006)
	ACGTATERD1	ACGT	−139	Nakashima et al. (2006)
	CACGTGMOTIF	CACGTG	−140	Busk and Pages (1998)
	MYB1AT	WAACCA	−659, −685	Abe et al. (2003)
	MYCCONSENSUSAT	CANNTG	−140	Abe et al. (2003)
	CRT/DRE	RYCGAC	−1005	Yamaguchi- Shinozaki and Shinozaki (1994)
	DPBFCOREDCDC3	ACACNNG	−141	Kim et al. (1997)
	MYB2AT	TAACTG	−173, −181	Urao et al. (1993)
	MYB2CONSENSUSAT	YAACKG	−87, −173, −181	Abe et al. (2003)

adverse environmental conditions since CaMIPS2 enzyme was shown to retain considerably higher activity than CaMIPS1 enzyme in in vitro condition in stress environments (Kaur et al. 2008). To investigate this, *CaMIPS1*, *CaMIPS2* and empty vector was transformed to *Arabidopsis thaliana*.

CaMIPS1 and *CaMIPS2*-transformed plants were initially screened for kanamycin resistance and subsequently confirmed by the GUS expression and PCR analysis. The transgenic lines which displayed 3 kan^R:1 kan^S segregation pattern in its progeny were selected for further analysis. Homozygous lines for each gene were identified and transcript expression of the transgenes was analyzed in several independent lines in T_3 generation. Higher but variable transcript accumulation of *CaMIPS1* and *CaMIPS2* was observed in various independent lines of respective transformants. For further analysis, lines with fairly similar *CaMIPS1* or *CaMIPS2* transcript accumulation were chosen among respective transgenics. Subsequently, inositol content was also estimated in these selected transgenics and enhanced accumulation of inositol was observed in *CaMIPS1* and *CaMIPS2*-transformed plants than empty vector control or wild-type plant (Fig. 4). Only two representative lines for each gene and empty vector-transformed lines as control have been shown in Fig. 4 and all subsequent figures. Noticeably, *CaMIPS2*-overexpressing lines were found to accumulate slightly more inositol than *CaMIPS1*-transformed lines though their transcript level was comparable. This observation was verified in several independent transgenic lines. Under normal growth conditions, *CaMIPS1* and *CaMIPS2* over expressing lines and empty vector-transformed lines were fairly comparable in growth such as plant height, fresh weight, number of silique or rosette diameter (Fig. 4d–g); however, both *CaMIPS1* and *CaMIPS2*-transformed seedlings displayed long root phenotype than wild type or empty vector-transformed plant at seedling stage. Interestingly, increased root length was more prominent in *CaMIPS2*-transformed lines (Fig. 4c).

Further, growth response of *CaMIPS1* and *CaMIPS2*-transformed lines were also evaluated under salinity stress. Initially seed germination was assessed in presence of increasing concentration of NaCl (0,100, 200 mM) and no significant differences of germination percentage, i.e., radicle emergence from seed were observed among wild type or vector control and *CaMIPS1* or *CaMIPS2*-transformed lines (data not shown). Differences became apparent during seedling development. At 0 mM NaCl, *CaMIPS1*, *CaMIPS2* and vector control transformed lines or wild type showed normal growth. However, at 200 mM NaCl, vector control or wild-type plants showed growth inhibition and turned yellow while both *CaMIPS1* and *CaMIPS2*-transformed seedlings displayed better growth response, developed the first true pair of leaves, remained green and retained considerable chlorophyll content than control plants. Interestingly, *CaMIPS2*-transformed seedlings showed better growth response and chlorophyll content than those transformed with *CaMIPS1* (Fig. 5a, b). To ascertain whether such stress responses are correlated with the accumulation of inositol in respective transgenic lines under stress condition, the level of inositol was estimated in respective transformants grown in presence of 200 mM NaCl for 10 days. Results showed that after prolonged exposure to salinity stress, inositol content was decreased in all lines with respect to normally grown seedlings; however, *CaMIPS1* and *CaMIPS2*-transformed plants were found to accumulate higher inositol than control plants. Interestingly, *CaMIPS2*-transformed lines which exhibited

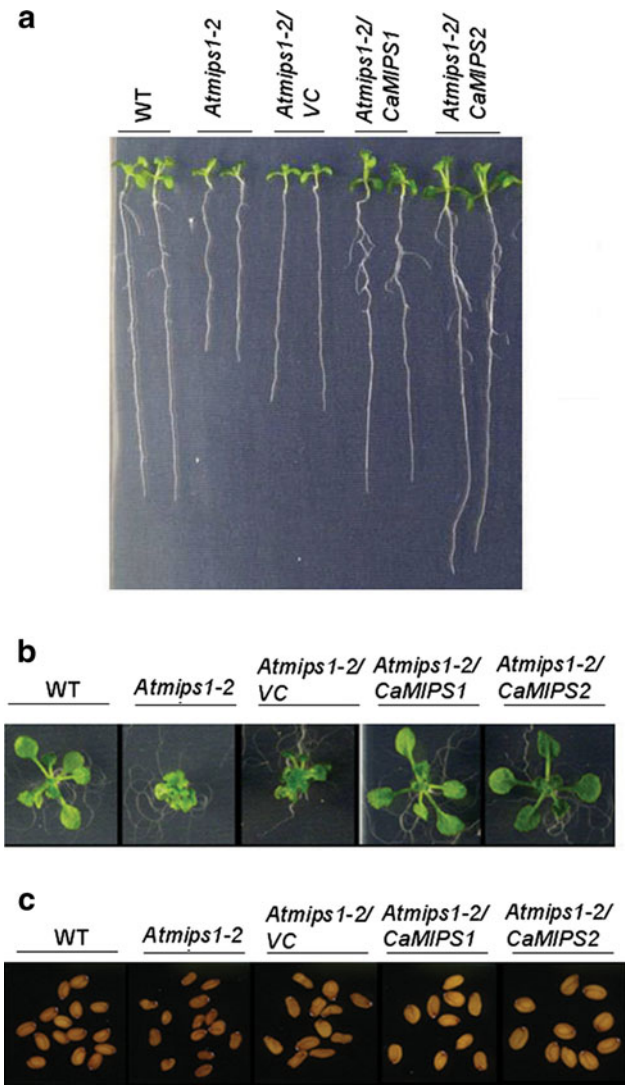

Fig. 3 Characterization of *Atmips1-2* mutant (SALK_023626) complemented by *CaMIPS1* and *CaMIPS2*. **a** Root phenotype of 12-day-old seedling of wild type (*WT*), *Atmips1-2*, and *Atmips1-2* harboring empty vector (*VC*), *CaMIPS1* or *CaMIPS2*. Seedlings were vertically grown in 1/2 MS0 agar medium. **b** Seedling phenotype. **c** Seed phenotype of wild type (*WT*), *Atmips1-2* and *Atmips1-2* harboring empty vector (VC), *CaMIPS1* or *CaMIPS2*. For seedling, photographs were taken at 12 days after germination. For assaying seed phenotype, all plants were grown in similar condition in culture room as described in "Materials and methods" section and seeds were harvested at the same time

better response towards stress were found to accumulate more inositol than *CaMIPS1* transformants in stress condition, even though the *CaMIPS1* and *CaMIPS2* transcript accumulation was same in respective transformed plants (Fig. 5c). To examine the basis of increased accumulation of inositol in *CaMIPS2*-transformed plants compared to *CaMIPS1* transformants particularly under stress conditions, the MIPS activity of these plants was assessed under normal and stress condition (200 mM NaCl). As expected, *CaMIPS1* and *CaMIPS2*-transformed lines grown in either normal or in stress condition exhibited higher MIPS activity than corresponding vector control or wild-type plants. However, MIPS activity in *CaMIPS2*-transformed plants was found to be always higher than *CaMIPS1*-transformed plants particularly in stress condition. It appeared that MIPS activity was less inhibited in stress condition and therefore *CaMIPS2*-transformed lines retained more activity than vector control or even *CaMIPS1*-transformed plants (Fig. 5d). Next, we investigated the growth pattern of these transformed plants in soil-grown mature plant under salinity stress. To analyze this, 14-day-old plants were transferred to pots and allowed to grow normally for two more weeks with routine watering. Then these 4-week-old plants were irrigated with 200 mM NaCl solution every alternate day for 12 days and growth pattern was monitored. Results showed that empty vector-transformed control plants developed chlorotic symptoms, turned yellow and started to die after 10 days of salt treatment. In contrast to this, *CaMIPS1* and *CaMIPS2* transgenic lines remained green and survived better than control plants (Fig. 6a). Transgenic plants were also assessed for their ability to withstand salt stress by considering several parameters (plant height, fresh weight, number of siliques and rosette diameter) of their growth potential under salt regime. Result revealed that in comparison to control plants, both *CaMIPS1* and *CaMIPS2* transgenic lines exhibited better response in these parameters. Moreover, *CaMIPS2* transgenic lines performed slightly better in all attributes when compared to *CaMIPS1* transgenic lines under such stress conditions (Fig. 6b–e).

Further, we investigated the growth pattern of transgenic mature plants under water-deficit condition. For this, 14-day-old plants were transferred to pots and allowed to grow normally for 10 more days with routine watering. Then these 24-day-old plants were subjected to water-deficit stress by withholding water for 3 weeks before the plants were watered again. Results revealed that empty vector, and *CaMIPS1* or *CaMIPS2* transgenic lines exhibited no growth differences when grown in normal routine watering condition. However, when plants were grown in absence of water, wild-type or empty vector-transformed plant showed more wilting symptoms as compared to *CaMIPS* transgenic lines. After rewatering, *CaMIPS2*-transformed plant recovered better and exhibited better growth response than the empty vector control plant or even *CaMIPS1*-transformed plants (Fig. 7a–e). The increased ability to survive after rewatering indicated that *CaMIPS2* transgenic plants are more drought tolerant over control plants or even *CaMIPS1* transgenic plants.

As a consequence of salinity and dehydration stress, oxidative stress is known to be induced as a secondary stress to plant. To investigate the tolerance of *CaMIPS1* and *CaMIPS2*-transformed plants towards oxidative stress, paraquat-

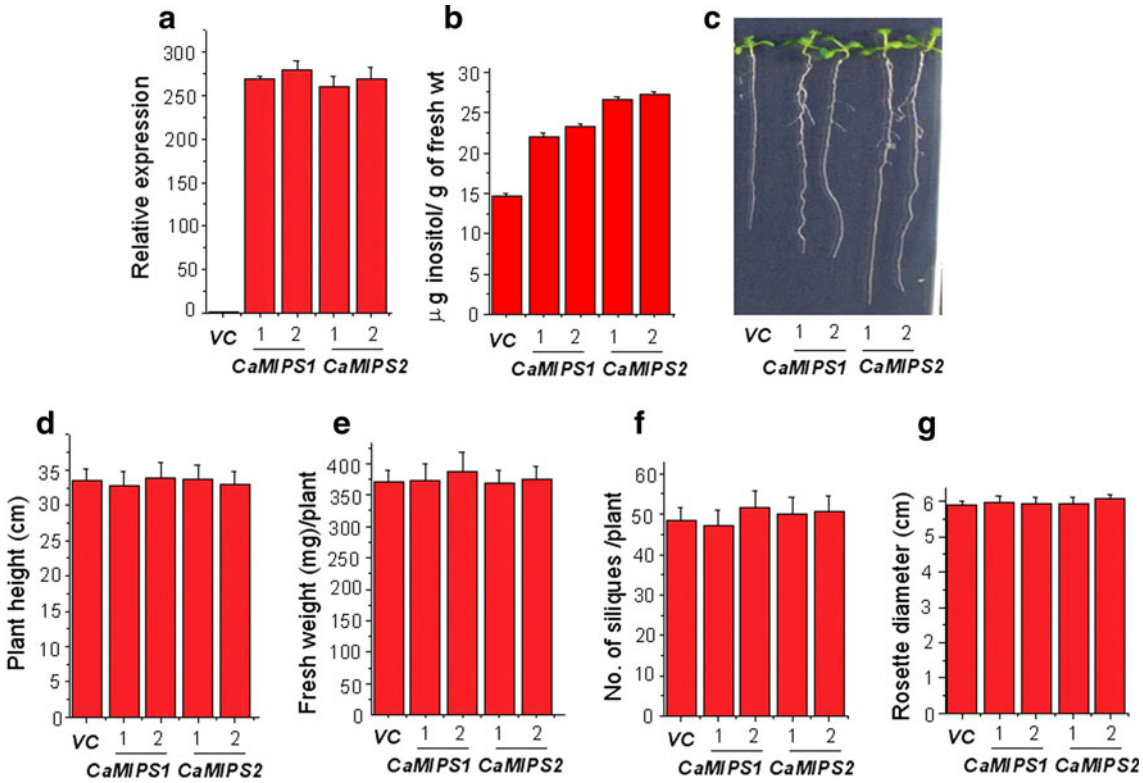

Fig. 4 Characterization of *Arabidopsis thaliana* overexpressing *CaMIPS1* and *CaMIPS2*. Only two representative independent lines (*1* line 1 and *2* line 2) of *CaMIPS1* and *CaMIPS2*-transformed plants are shown here. As a control set, both wild type and empty vector-transformed lines were used but only empty vector-transformed lines are shown here as a representative control. **a** Transcript accumulation of *CaMIPS1* and *CaMIPS2* in respective transgenic lines as determined by quantitative real-time PCR. 18SrRNA was used as an endogenous control. Only two representative independent transformed lines for each gene exhibiting fairly equal *CaMIPS1* or *CaMIPS2* transcript accumulation are shown here and were used for subsequent analysis. Values are the result of triplicate analysis of three biological replicates. *Error bars* indicate the standard deviation. **b** Inositol content of respective transgenic lines. Two-week-old seedlings in each case were used to determine the inositol level by gas–liquid chromatography as described in "Materials and methods" section. Inositol levels are expressed as microgram per gram of fresh weight. Values are the result of triplicate analysis. *Error bars* indicate the standard deviation. **c** Comparison of seedling growth among empty vector (*VC*), *CaMIPS1* and *CaMIPS2* transformed lines. 12-day-old seedlings were vertically grown in 1/2 MS0-agar plates. **d**–**g** Comparison of growth responses of soil-grown transgenic lines: **d** plant height, **e** fresh weight, **f** number of siliques per plant, **g** rosette diameter. Data represent the mean ± SD and was obtained from 20 independent plants per line

induced leaf bleaching experiment was conducted. Paraquat is an oxidative stress inducer herbicide which is known to cause chlorophyll loss and leaf bleaching because of its ability to increase H_2O_2 production in the mesophyll cells (Asada 1996; Sunkar et al. 2003). A distinct visual difference of bleaching in leaf due to the loss of chlorophyll was observed among vector control plants and different transgenic lines. Leaf bleaching was pronounced in empty vector-transformed plant or wild type than the *CaMIPS1* and *CaMIPS2* transformed plants (Fig. 7f). Further, chlorophyll content was also measured in paraquat-treated leaves of *CaMIPS1*, *CaMIPS2* and vector-transformed plants. Results revealed that in *CaMIPS1* and *CaMIPS2*-transformed lines, oxidative stress-induced chlorophyll loss was less compared to vector control lines and *CaMIPS2* lines appeared to be more tolerant towards this oxidative stress in comparison to *CaMIPS1* transgenic lines (Fig. 7g).

Discussion

Inositol has been shown to be an essential component for plant growth and development; therefore, plants maintain inositol pool at a basal level throughout their life cycle (Loewus and Murthy 2000). However, not all but few stress-tolerant plants like ice plant or chickpea were shown to elevate their inositol level in response to environmental stresses, possibly to meet additional demand of inositol that might be required for their adaptation to stresses (Ishitani et al. 1996; Boominathan et al. 2004). In addition, few extremophiles and stress-tolerant plants encode unique MIPS proteins which are able to retain their catalytic activity even in high temperature or high salt concentration, probably to synthesize inositol even under stress situation in unconstrained manner (Chen et al. 2000; Majee et al. 2004; Dastidar et al. 2006). Such properties are suggested to arise

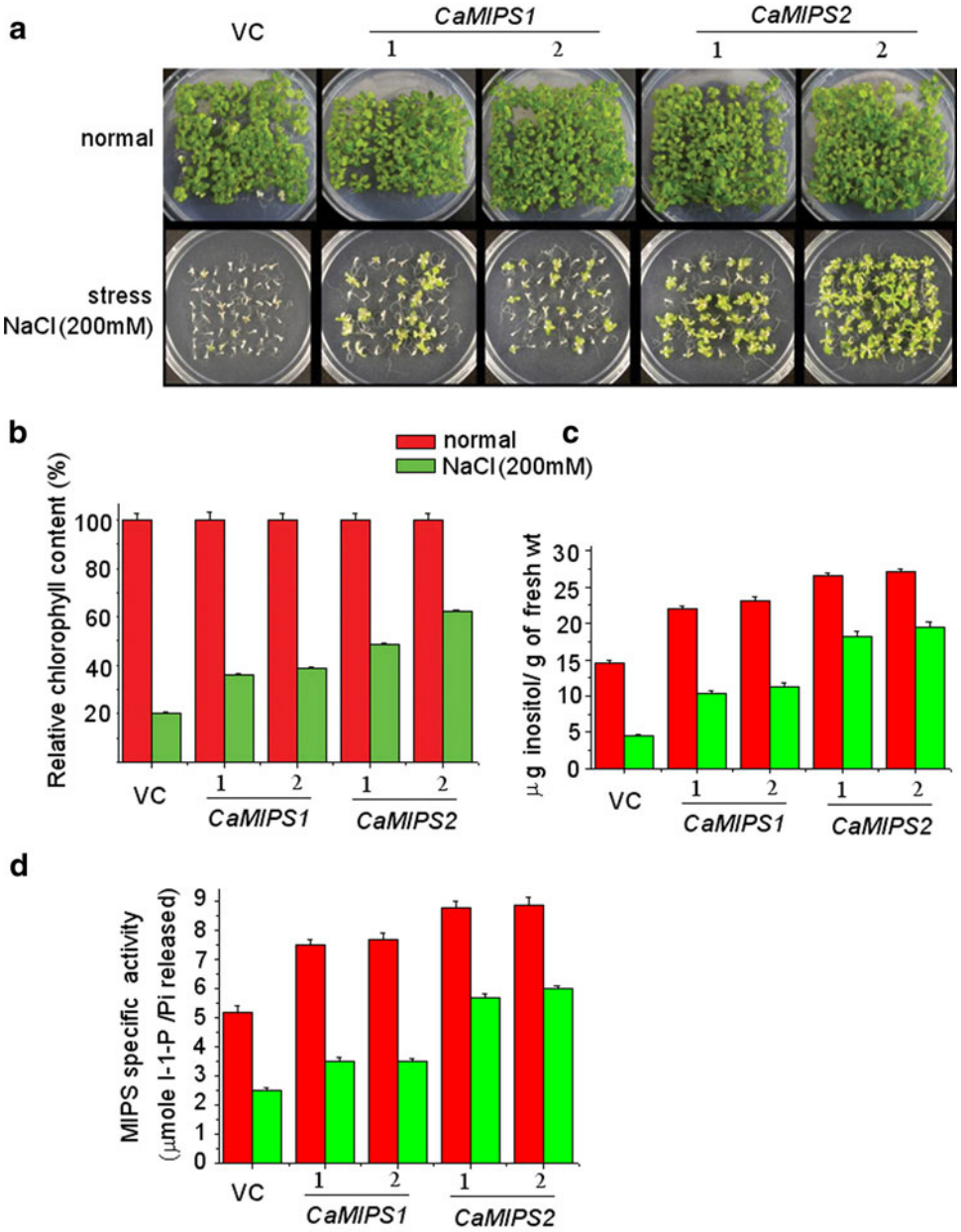

Fig. 5 **a** Growth pattern of *CaMIPS1, CaMIPS2* and empty vector-transformed seedling under normal (*upper panel*) and saline environment (200 mM NaCl) (*lower panel*). Only two representative independent lines (*1* line1 and *2* line 2) of *CaMIPS1* and *CaMIPS2*-expressing plants are shown here. As a control set, both wild type and empty vector-transformed lines were used but only empty vector-transformed lines are shown here as a representative control. Seeds were germinated and continued to grow in 1/2 MS0-agar plate with or without 200 mM NaCl, Photographs were taken at 10 days after germination. **b** Relative chlorophyll content of the seedlings grown in normal and salt (200 mM NaCl) containing medium. Total chlorophyll was quantified in seedling (10 days after germination) grown in presence and absence of 200 mM salt as described in "Materials and methods" section. Total chlorophyll content in all lines grown in absence of salt (control) was in the range of 775–800 microgram per gram of fresh weight and used as 100 % for each. Values are the result of triplicate analysis. *Error bars* indicate the SD. **c** Total inositol content of the seedlings grown on normal and salt containing (200 mM NaCl) medium. Inositol was quantified as described previously. Values are the result of triplicate analysis. *Error bars* indicate the standard deviation. **d** Total MIPS activity of the seedlings grown on normal and salt containing (200 mM NaCl) medium. ~50 µg of total protein was used for the enzyme assay and specific activity was calculated as micromoles of inositol 1-phosphate released per mg per hour. Values are the result of three independent experiments. *Error bars* indicate the SD

due to adaptive changes in their coding sequences, depending on the environment they live in. As for example, wild halophytic rice is reported to encode salt-tolerant MIPS, while cultivated rice codes for salt-sensitive MIPS (Majee et al. 2004). In this study, we have chosen chickpea, since chickpea is relatively drought-tolerant plant which requires

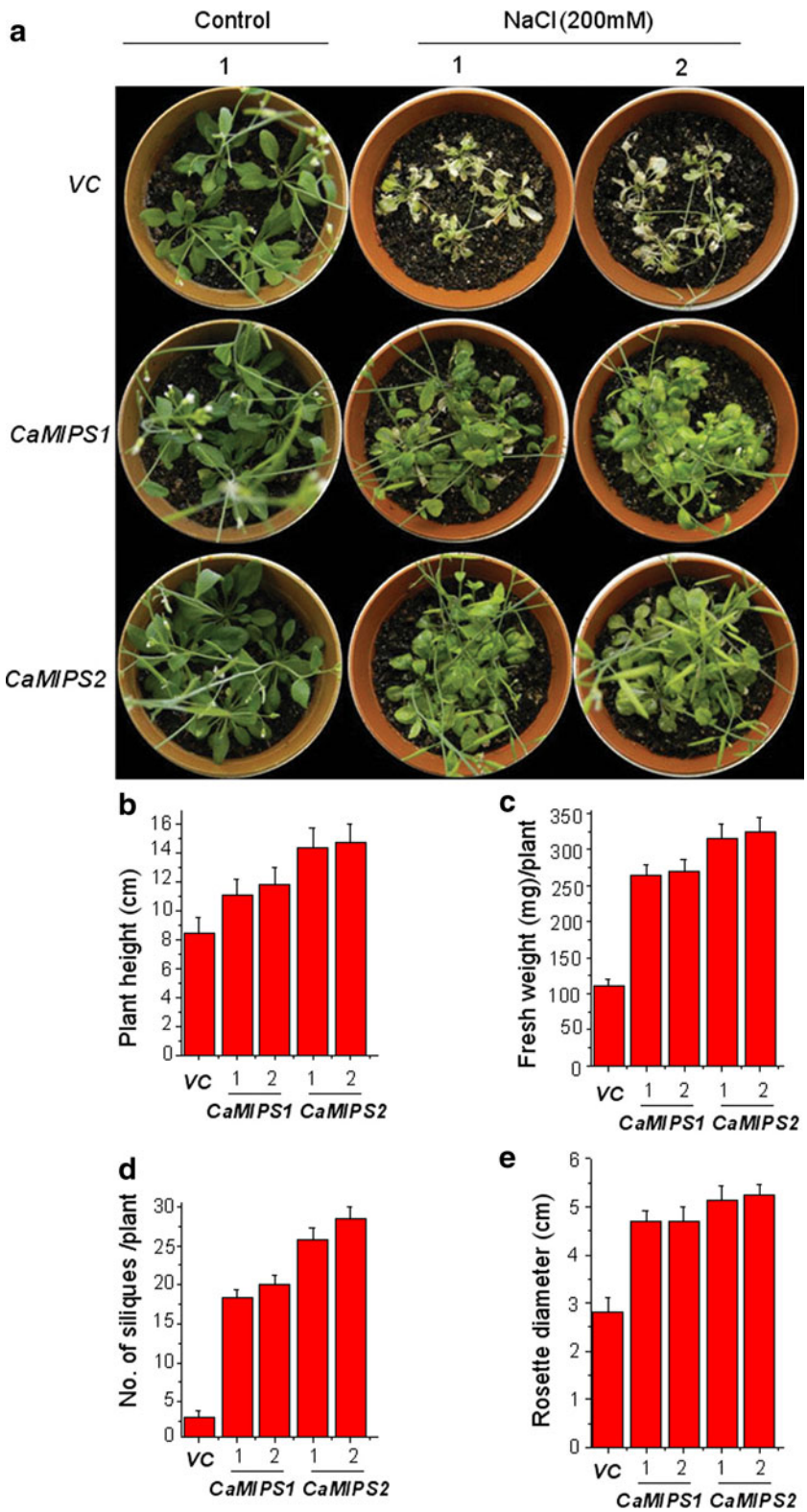

Fig. 6 **a** Growth pattern of *CaMIPS1, CaMIPS2* and empty vector-transformed mature soil-grown plant under normal and saline condition (200 mM NaCl). Only two representative independent lines (*1* line 1 and *2* line 2) of *CaMIPS1* and *CaMIPS2*-transformed plants are shown here. As a control set, only empty vector-transformed lines are shown here as representative control. **b**–**e** Comparative analyses of growth responses of transgenic lines under salt stress: **b** plant height, **c** fresh weight, **d** number of siliques per plant, **e** rosette diameter. Data represent the mean ± SD and was obtained from 20 independent plants per line

less irrigation and adapts well in water-limited environment. Apart from its immense agronomic and nutritional importance, long-term evolution and adaptation to harsh environmental conditions make chickpea rich in resistant genes for various stress tolerance (Turner et al. 2001). In our previous study, we reported two differentially expressed divergent genes (*CaMIPS1* and *CaMIPS2*) which encode two MIPS isoforms with distinct biochemical properties (Kaur et al.

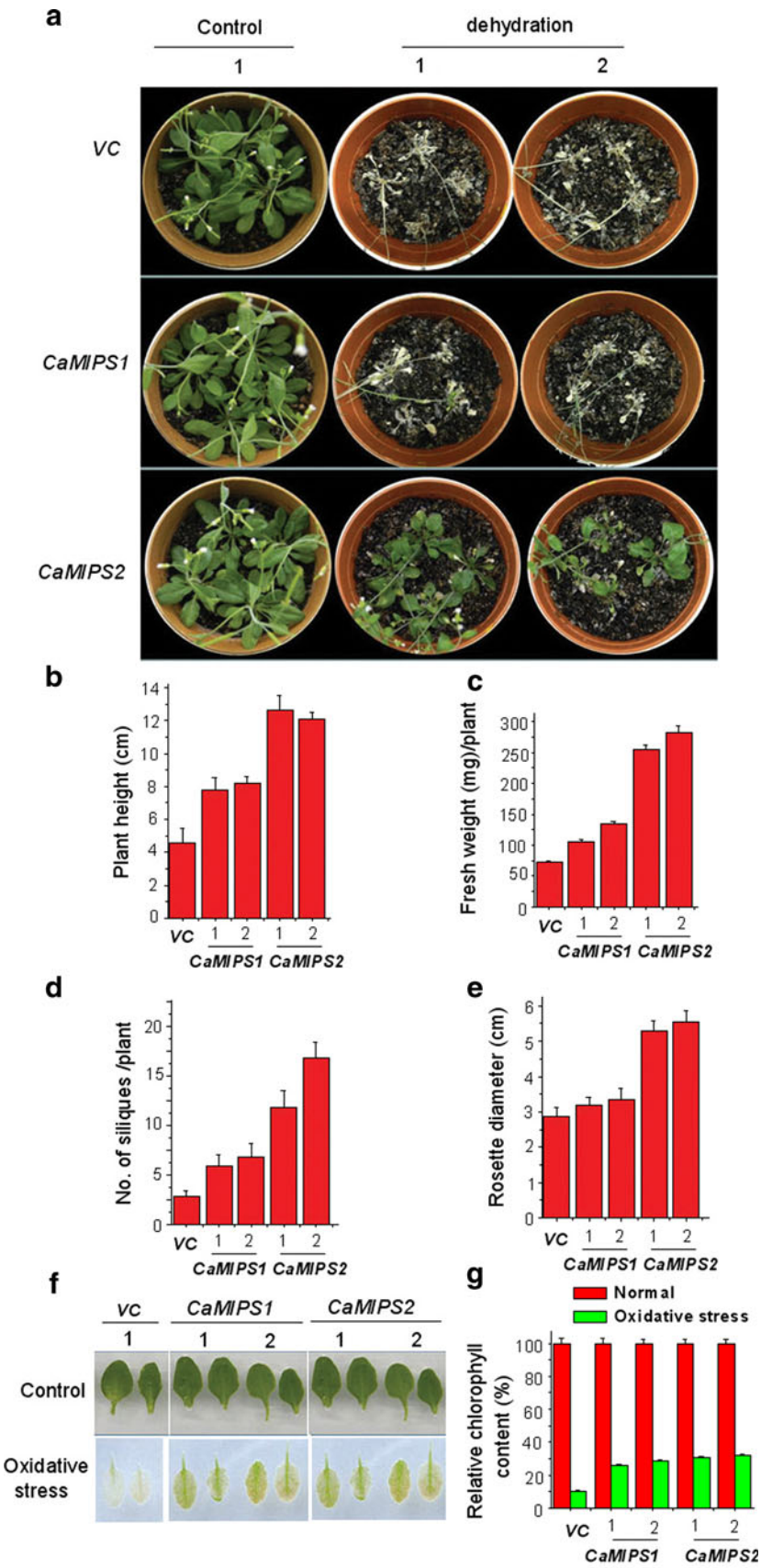

Fig. 7 **a** Growth pattern of *CaMIPS1*, *CaMIPS2* and empty vector-transformed mature soil-grown plants under normal and water-deficit condition (photographs were taken on the fifth day after rewatering). **b–e** Comparative analyses of growth responses of transgenic lines under water deficit condition: **b** plant height, **c** fresh weight, **d** number of siliques per plant, **e** rosette diameter. Data represent the mean ± SD and was obtained from 20 independent plants per line. **f** Effect of paraquat on leaf bleaching of *CaMIPS1, CaMIPS2* and empty vector-transformed lines. Leaves of 4-week-old mature plants from *CaMIPS1* and *CaMIPS2* overexpression lines and vector control plants were treated with 10 µM of paraquat for 24 h in continuous light. **g** Relative chlorophyll content in normal and paraquat-treated (10 µM) leaf of *CaMIPS1, CaMIPS2* and empty vector-transformed lines. Values are the result of triplicate analysis and were obtained from 20 independent plants per line. *Error bars* indicate the SD. Total chlorophyll content in normal condition is used as 100 %. In each result, only two representative independent lines (*1* line1 and *2* line 2) of *CaMIPS1* and *CaMIPS2*-transformed plants are shown. As a control set, only empty vector-transformed lines are shown here as a representative control

2008). In this study, the experimental data suggest that in contrast to *CaMIPS1*, Ca*MIPS2* is an early dehydration-responsive gene in chickpea and the dehydration-induced expression appears to be mediated by ABA-dependent pathway (Fig. 1). Unlike this, in *Mesembryanthemum crystallinum, MIPS* gene was shown to be induced by salinity stress but not by drought stress or ABA. Likewise, in yellow passion fruit, *PeMIPS1* was shown to be differentially regulated in heat and cold stress, while stress-sensitive plant species like *Arabidopsis thaliana* do not show any significant upregulation of MIPS transcript under any stress condition (Vernon et al. 1993; Ishitani et al. 1996). These reports indicate that the regulation of expression of MIPS genes seems to be species specific and also dependent on the stress-responsive nature of the species. Besides stress, ABA-induced expression of MIPS in bud or seed was shown in many plant species (Smart and Fleming 1993; Yoshida et al. 1999). Promoter analyses of *CaMIPS1* and *CaMIPS2* revealed that differential promoter activity is possibly responsible for the differential expression of these two genes and the CRT/DRE *cis*-regulatory element is likely to play important role in dehydration-induced *CaMIPS2* expression (Fig. 2). Interestingly, dehydration-induced *CaMIPS2* promoter-driven GUS transcript accumulation in Arabidopsis was found to be lesser than in its native system chickpea, probably because *CaMIPS2* promoter-driven transcriptional activation differs in orthologous system, i.e., Arabidopsis than its native plant chickpea. Although, the influence of 3′UTR or intron cannot be ruled out for this dehydration-induced *CaMIPS2* expression in chickpea, as UTR and intron region have been shown to affect gene expression in few plants (Callis et al. 1987; Rose et al. 2008).

Subsequent experimental data suggests that both *CaMIPS1* and *CaMIPS2* are functional in Arabidopsis plant, as their expression in Arabidopsis *Atmips1* knockout mutant plant could restore the defect of seedling growth and wrinkled seed phenotype. Reduced inositol content has previously been shown to affect the plants growth and development and makes them susceptible to various environmental stresses (Keller et al. 1998; Nunes et al. 2006; Meng et al. 2009; Donahue et al. 2010; Luo et al. 2011). Even though few plants are known to accumulate increased inositol as early response to stresses, high salinity and prolonged exposure to stress often results in reduced accumulation of inositol (Ishitani et al. 1996; Boominathan et al. 2004; Majee et al. 2004). Our results suggest that stress-induced growth inhibition of Arabidopsis control plant was associated with reduced level of inositol, while transformed lines accumulating higher inositol displayed better growth responses under various stress conditions. However, when grown in normal environment, we did not see any apparent advantage to the transgenics with higher accumulation of inositol, except slightly long root phenotype at seedling stage. Interestingly, *CaMIPS2*-transformed lines exhibited better growth with improved traits than *CaMIPS1*-transformed lines under stress conditions particularly in dehydration stress. Subsequent analysis confirmed that *CaMIPS2*-transformed lines were able to produce considerably more inositol due to the higher MIPS activity in stress situations and thus provided better growth response in stress environment (Fig. 5). This observation can be justified in the light of our previous work where we have shown that CaMIPS2 enzyme is catalytically more active than CaMIPS1 enzyme in stress situations due to the difference of amino acid residues between CaMIPS1 and CaMIPS2 protein. Further through bioinformatics study, CaMIPS2 was shown to be structurally more stable and thus retain more activity than CaMIPS1 in presence of destabilizing agents like high salt, temperature, etc. (Kaur et al. 2008). Our results also match closely with earlier studies (Majee et al. 2004; Das Chatterjee et al. 2006) where salt-tolerant character of *PcINO1* (MIPS-coding gene from halophytic wild-rice *Porteresia coarctata*) gene product provides better tolerance to plants over salt-sensitive *OsINO1* (MIPS-coding gene from salt-sensitive *Oryza sativa*) due to increased synthesis of inositol by the former in stress conditions. Under stress conditions, the plants transformed with such stress-tolerant enzymes are only able to maintain required inositol level, which is essential even under stress condition to maintain plants growth and development. Additionally, inositol serves as a precursor or substrate for many plant metabolites like phosphatidylinositol, ascorbic acid, galactinol, etc. Among these metabolites, galactinol or ascorbic acid is known to act as ROS scavenger and play a significant role in abiotic stress tolerance (Nishizawa et al. 2008). Recently, *Atmips1* mutant, defective in inositol biosynthesis was reported to contain reduced galactinol along with inositol and such deficiencies were shown to be associated with spontaneous cell death. In our study, we observed enhanced tolerance towards paraquat-induced oxidative stress in leaf of *CaMIPS* transformed plants; however, further analysis will be required to understand the inositol-mediated cellular processes for such oxidative stress tolerance. Together, our results are in agreement with the assumption that enhanced stress tolerance of MIPS-overexpressing lines were due to the increased level of inositol which might result in the possible increment of inositol derivatives like galactinol, or ascorbic acid, etc.

Collectively, our results strongly suggest that *CaMIPS2* is an ABA-inducible, dehydration-responsive gene in chickpea and has possibly evolved through gene duplication followed by adaptive changes in 5′ upstream regulatory sequences and protein coding sequences to express and function better under harsh environmental conditions and play a key role in stress adaptation in chickpea besides its role in growth and development.

Acknowledgments This work was supported by the grant (BT/PR12919/AGR/02/676/2009) from Department of Biotechnology, Government of India. H.K. B.P, V.R and P.V. thank Council of Scientific and Industrial Research and University Grant Commission, Government of India, for research fellowships. We are grateful to Dr. Glenda Gillaspy, Virginia Tech., USA, for providing us the *Atmips1* mutant (*Atmips1-2,* SALK_023626). We are grateful to Dr. S.K. Pattanaik, University of Kentucky, for providing pKYLX 70:GUS vector.

References

Abe H, Urao T, Ito T, Seki M, Shinozaki K, Yamaguchi-Shinozaki K (2003) Arabidopsis AtMYC2 (bHLH) and AtMYB2 (MYB) function as transcriptional activators in abscisic acid signaling. Plant Cell 15:63–78

Abreu EFM, Aragao FJL (2007) Isolation and characterization of a *myo*-inositol phosphate synthase gene from yellow passion fruit (*Passiflora edulis f. flavicarpa*) expressed during seed development and environmental stress. Ann Bot Lond 99:285–292

Ahmad F, Gaur PM, Croser J (2005) Chickpea (*Cicer arietinum* L.) In: Singh R, Jauhar P (eds) Genetic resources, chromosome engineering and crop improvement- grain legumes, vol 1. CRC Press, USA, pp 187–217

Arnon DL (1949) Copper enzymes in isolated chloroplasts: polyphenol oxidase in *Beta vulgaris*. Plant Physiol 24:1–15

Asada K (1996) Radical production and scavenging in the chloroplasts. In: Baker NR (ed) Photosynthesis and the environment. Kluwer, Dordrecht, pp 123–150

Barnett JEG, Brice RE, Corina DL (1970) A colorimetric determination of inositol monophosphatase as an assay for D-glucose 6-phosphate 1-L *myo*-inositol 1-phosphate cyclase. Biochem J 119:183–186

Bieleski RL, Redgwell RJ (1977) Synthesis of sorbitol in apricot leaves. Aust J Plant Physiol 4:1–10

Boominathan P, Shukla R, Kumar A, Manna D, Negi D, Verma PK, Chattopadhyay D (2004) Long term transcript accumulation during the development of dehydration adaptation in *Cicer arietinum*. Plant Physiol 135:1608–1620

Bradford MW (1976) Quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Anal Biochem 72:248–254

Busk PK, Pages M (1998) Regulation of abscisic acid-induced transcription. Plant Mol Biol 37:425–435

Callis J, Fromm M, Walbot V (1987) Intron increases gene expression in cultured maize cells. Genes Dev 1:1183–1200

Chaouch S, Noctor G (2010) *Myo* inositol abolishes salicylic acid dependent cell death and pathogen defense responses triggered by peroxisomal hydrogen peroxide. New Phytol 188:711–718

Chen RS, Toribara TY, Warner H (1956) Microdetermination of phosphorus. Anal Chem 28:1756–1758

Chen L, Zhou C, Yang H, Robert MF (2000) Inositol 1-phosphate synthase from *Archaeoglobus fulgidus* is a class II aldolase enzyme. Biochemistry 39:12415–12423

Chun JA, Jin UH, Lee JW, Yi YB, Hyung NI, Kang MH, Pyee JH, Suh MC, Kang CW, Seo HY, Lee SW, Chung HC (2003) Isolation and characterization of a *myo*-inositol 1-phosphate synthase cDNA from developing sesame (*Sesamum indicum* L.) seeds: functional and differential expression, and salt induced transcription during germination. Planta 216:874–880

Clough SJ, Bent AF (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. Plant J 16:735–743

Das Chatterjee A, Goswami L, Maitra S, Dastidar KG, Ray S, Majumder AL (2006) Introgression of a novel salt tolerant L-*myo*-inositol-1-phosphate synthase from *Porteresia coarctata* (Roxb.) Tateoka (*PcINO1*) confers salt tolerance to evolutionary diverse organisms. FEBS Lett 580:3980–3988

Dastidar KG, Maitra S, Goswami L, Roy D, Das KP, Majumder AL (2006) An insight into the molecular basis of salt-tolerance of L-*myo*-inositol 1-phosphate synthase (*PcINO1*) from *Porteresia coarctata* (Roxb.) Tateoka, a halophytic wild rice. Plant Physiol 140:1279–1296

Donahue JL, Alford SR, Torabinejad AJ, Kerwin RE, Nourbakhsh A, Ray K, Hernick M, Huang X, Lyons BM, Hein PP, Gillaspy GE (2010) The *Arabidopsis thaliana myo*-inositol-1-phosphate synthase 1 gene is required for *myo* inositol synthesis and suppression of cell death. Plant Cell 22:888–903

Hegeman CE, Good LL, Grabau EA (2001) Expression of D-*myo*-inositol-3-phosphate synthase in soybean. Implications for phytic acid biosynthesis. Plant Physiol 125:1941–1948

Higo K, Ugawa Y, Iwamoto M, Korenaga T (1999) Plant *cis* acting regulatory DNA elements (PLACE) database. Nucleic Acid Res 27:297–300

Ishitani M, Majumder AL, Bornhouser A, Michalowski CB, Jensen RG, Bohnert HJ (1996) Coordinate transcriptional induction of *myo*-inositol metabolism during environmental stress. Plant J 9:537–548

Jefferson RA, Kavanagh TA, Bevan MW (1987) GUS fusion: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J 6:3901–3907

Kaur H, Shukla RK, Yadav G, Chattopadhyay D, Majee M (2008) Two divergent genes encoding L-*myo*-inositol 1-phosphate synthase 1 (*CaMIPS1*) and 2 (*CaMIPS2*) are differentially expressed in chickpea. Plant, Cell Environ 31:1701–1716

Keller R, Brearley CA, Trethewey RN, Müller-Röber B (1998) Reduced inositol content and altered morphology in transgenic potato plants inhibited for 1D-*myo*-inositol 3-phosphate synthase. Plant J 16:403–410

Kim SY, Chung HJ, Thomas TL (1997) Isolation of a novel class of bZIP transcription factors that interact with ABA-responsive and embryo-specification elements in the Dc3 promoter using a modified yeast one-hybrid system. Plant J 11:1237–1251

Lescot M, Dehais P, Thijs G, Marchal K, Moreau Y, Van der Peer Y, Rouze P, Rombauts S (2002) PlantCARE, a database of plant cis acting regulatory elements and a portal of tools for in silico analysis of promoter sequences. Nucleic Acid Res 30:325–327

Loewus FA (1990) Inositol biosynthesis. In: Morre DJ, Boss WF, Loewus FA (eds) Inositol metabolism in plants. Wiley-Liss, New York, pp 13–19

Loewus FA, Loewus MW (1983) *Myo*-inositol: its biosynthesis and metabolism. Annu Rev Plant Physiol 34:137–161

Loewus FA, Murthy PN (2000) *Myo*-inositol metabolism in plants. Plant Sci 150:1–19

Luo Y, Qin G, Zhang J, Liang Y, Song Y, Zhao M, Tsuge T, Aoyama T, Liu J, Gu H, Qu LJ (2011) D-*myo*-inositol-3-phosphate affects phosphatidylinositol-mediated endomembrane function in Arabidopsis and is essential for auxin-regulated embryogenesis. Plant Cell 23:41352–41372

Majee M, Maitra S, Dastidar KG, Pattnaik S, Chatterjee A, Hait NC, Das KP, Majumder AL (2004) A novel salt tolerant L *myo*-inositol-1-phosphate synthase from *Porteresia coarctata* (Roxb.) Tateoka, a halophytic wild rice: molecular cloning, bacterial overexpression, characterization and functional introgression into tobacco conferring salt tolerant phenotype. J Biol Chem 279:28539–28552

Majumder AL, Chatterjee A, Dastidar KG, Majee M (2003) Diversification and evolution of L-*myo*-inositol 1 phosphate synthase. FEBS Lett 533:3–10

Meng HP, Raynaud C, Tcherkez G, Blanchet S, Massoud K, Domenichini S, Henry Y, Soubigou-Taconnat L, Lelarage-Trouverie C,

Saindrenan P, Renou JP, Bergounioux C (2009) Crosstalk between *myo*-inositol metabolism, programmed cell death and basal immunity in *Arabidopsis*. Plos One: e7364

Nakashima K, Fujita Y, Katsura K, Maruyama K, Maruyama K, Narusaka Y, Seki M, Shinozaki K, Yamaguchi-Shinozaki K (2006) Transcriptional regulation of AB13 and ABA responsive genes including RD29B and RD29A in seeds, germinating embryos and seedlings of Arabidopsis. Plant Mol Biol 60:51–68

Nelson DE, Rammesmayer G, Bohnert HJ (1998) Regulation of cell specific inositol metabolism and transport in plant salinity tolerance. Plant Cell 10:753–764

Nishizawa A, Yabuta Y, Shigeoka S (2008) Galactinol and raffinose constitute a novel function to protect plants from oxidative damage. Plant Physiol 147:1251–1263

Nunes ACS, Vianna GR, Cuneo F, Amaya-Farfa'n J, Capdeville G, Rech EL, Aragao FJL (2006) RNAi mediated silencing of the *myo*-inositol-1-phosphate synthase gene (*GmMIPS1*) in transgenic soybean inhibited seed development and reduced phytate content. Planta 224:125–132

Paul MJ, Cockburn W (1989) Pinitol, a compatible solute in *Mesembryanthemum crystallinum* L. J Exp Bot 40:1093–1098

Qian W, Yu C, Qin H, Xin L, Zhang A, Johansen IE, Wang D (2007) Molecular and functional analysis of phosphomannomutase (PMM) from higher plants and genetic evidence for the involvement of PMM in ascorbic acid biosynthesis in *Arabidopsis* and *Nicotiana benthamiana*. Plant J 49:399–413

Rose AB, Elfresh T, Parra G, Korf I (2008) Promoter proximal introns in *Arabidopsis thaliana* are enriched in dispersed signals that elevate gene expression. Plant Cell 20:543–551

Shen B, Hohmann S, Jensen RG, Bohnert HJ (1999) Roles of sugar alcohols in osmotic stress adaptation. Replacement of glycerol by mannitol and sorbitol in yeast. Plant Physiol 121:45–52

Sheveleva E, Chmara W, Bohnert HJ, Jensen RG (1997) Increased salt and drought tolerance by D-ononitol production in transgenic *Nicotiana tabaccum* L. Plant Physiol 115:1211–1219

Smart CC, Fleming AJ (1993) A plant gene with homology to D-*myo*-inositol-3 phosphate synthase is rapidly and spatially upregulated during an abscisic acid induced morphogenic response in *Spirodela polyrrhiza*. Plant J 4:279–293

Stevenson JM, Perera IY, Heilmann I, Persson S, Boss WF (2000) Inositol signaling and plant growth. Trends Plant Sci 5:252–258

Sunkar R, Bartels D, Kirch H (2003) Overexpression of a stress inducible aldehyde dehydrogenase gene from *Arabidopsis thaliana* in transgenic plants improves stress tolerance. Plant J 35:452–464

Syam Prakash SR, Jayabaskaran C (2006) Expression and localization of calcium-dependent protein kinase isoforms in chickpea. J Plant Physiol 163:1135–1149

Taji T, Ohsumi C, Iuchi S, Seki M, Kasuga M, Kobayashi M, Yamaguchi-Shinozaki K, Shinozaki K (2002) Important roles of drought and cold inducible genes for galactinol synthase in stress tolerance in *Arabidopsis thaliana*. Plant J 29:417–426

Turner NC, Ma Q, Leport L, Davies SL, Siddique KHM (2001) Adaptation of chickpea in water limited environment. In: Proceedings of the 10th Australian Agronomy Conference, January 29–February 1, 2001, Hobart, Australia

Urao T, Yamaguchi-Shinozaki K, Urao S, Shinozaki K (1993) An Arabidopsis *myb* homolog is induced by dehydration stress and its gene product binds to the conserved MYB recognition sequence. Plant Cell 5:1529–1539

Vernon DM, Tarczynski MC, Jensen RG, Bohnert HJ (1993) Cyclitol production in transgenic tobacco. Plant J 4:199–205

Wellburn AR, Lichtenthaler (1984) Formulae and program to determine total carotenoids and chlorophyll *a* and *b* leaf extracts in different solvents. In: Sybesma C (ed) Advances in photosynthesis research, vol 2., Martinus Nijhoff/DrW. Junk, The Hague, pp 9–12

Yamaguchi-Shinozaki K, Shinozaki K (1994) A novel *cis*-acting element in an Arabidopsis gene is involved in responsiveness to drought, low temperature or high salt stress. Plant Cell 6:251–264

Yoshida KT, Wada T, Koyama H, Mizobuchi-Fukuoka R, Naito S (1999) Temporal and spatial patterns of accumulation of the transcript of *myo*-inositol-1-phosphate synthase and phytin containing particles during seed development in rice. Plant Physiol 119:65–72