A putative tomato inositol polyphosphate 5-phosphatase, Le5PT1, is involved in plant growth and abiotic stress responses

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Received: 21 November 2018 / Accepted: 20 December 2019 / Published online: 4 January 2020
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Abstract

Based on sequence similarity to *Arabidopsis* inositol polyphosphate 5-phosphatases (5PTases) involved in abiotic stress responses and development, four tomato cDNAs (*Le5PT1-4*) encoding putative 5PTase proteins were identified. The predicted protein sequences of the Le5PTs include conserved catalytic domains required for 5PTase enzyme activity. Le5PT1, 2, and 3 showed high amino acid sequence identity with At5PTase2, At5PTase1 and At5PTase3, and At5PTase5 and At5PTase6, respectively. The expression of *Le5PT1* was downregulated soon after initiation of dehydration and salt stress as well as exposure to polyethylene glycol (PEG) and NaCl, but not by exogenous ABA treatment. On the other hand, the expression of *Le5PT2* gradually increased with time in all treatments. Transgenic tobacco plants overexpressing *Le5PT1* exhibited reduced growth in height, leaf area, and dry weight compared to wild type plants. Transgenic plants also had lower water use efficiency (WUE) than wild type and the downregulation of the drought-responsive gene, *NtERD10B*. Together these results suggest that Le5PT1 may have a negative role in response to water deficit through the repression of drought-inducible genes that in turn affects plant growth and development.

Keywords Drought · *At5PTase* · Type I inositol 5 phosphatases · *Le5PT1* · *NtERD10B*

Introduction

Plants respond to environmental stresses through signaling cascades that alter the expression of stress-responsive genes, which play important roles in adaptation and survival under adverse conditions. Inositol 1,4,5 triphosphate (InsP₃) acts as a second messenger not only in various abiotic and biotic stress responses in plants, but also during plant growth and developmental events such as gravitropic growth (Perera et al. 2006; Salinas-Mondragon et al. 2010), pollen

tube growth (Monteiro et al. 2005), hypocotyl elongation (Gunsekera et al. 2007), and pollen dormancy and germination (Wang et al. 2012). InsP₃ levels increase in response to various abiotic stimuli (Im et al. 2010). In maize pulvini, the level of InsP₃ changes rapidly in response to gravistimulation (Perera et al. 1999), which is followed by gravitropic growth. Pollen tube growth was also observed after release of caged InsP₃ in *Agapanthus umbellatus* (Gunsekera et al. 2007; Monteiro et al. 2005). Given its role in multiple stresses and developmental pathways, levels of InsP₃ are tightly regulated by balancing InsP₃ synthesis and degradation to modulate proper spatial and temporal responses (Im et al. 2010).

In plants, InsP₃ is generated via hydrolysis of phosphatidylinositol (4,5) bisphosphate (PtdInsP₂) by phospholipase C (PLC) (Mueller-Roeber and Pical 2002), which is a major route of InsP₃ synthesis in plants, although there are alternate pathways for InsP₃ production, such as those involving phosphorylation of Ins(1,4)P₂ and Ins(1,5)P₂ (Im et al. 2010). *Arabidopsis* *PLC1* expression is upregulated by a variety of environmental stresses (Hirayama et al. 1995), which in turn increase InsP₃ levels (DeWald et al. 2001; Ruelland et al. 2002; Sanchez and Chua 2001; Takahashi et al. 2001; Xiong et al. 2002). On the other hand, InsP₃

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s13205-019-2023-y>) contains supplementary material, which is available to authorized users.

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can be metabolized through phosphorylation by triphosphate 3-kinase (Im et al. 2010; Takazawa et al. 1991) or dephosphorylation by type I 5PTases (Chen et al. 2008; Im et al. 2010; Majerus et al. 1999). In plants, InsP_3 can be degraded by both type I 5PTases (Berdy et al. 2001; Sanchez and Chua 2001) and inositol polyphosphate 1-phosphatases (1PTases) (Xiong et al. 2001); the removal of either phosphate terminates signaling through InsP_3 . The *Arabidopsis* genome contains at least 15 5PTases (Heilmann 2016), most of which have shown to dephosphorylate 5' phosphate in the inositol ring of InsP_3 or phosphatidyl inositol (Gillaspy 2011). Zhang et al. (2019) reported that the presence of multiple Type I 5PTases with different functions in plants resulted likely from a gene duplication event followed by gaining different functions through diverged substrate specificity of each Type I 5PTase.

It is known that InsP_3 levels increase in response to environmental stresses and that InsP_3 injection into guard cells elevates intracellular Ca^{2+} concentrations to mediate ABA-induced stomatal closure. These findings suggested that altered InsP_3 levels might affect drought responses in plants (Blatt et al. 1990; Gilroy et al. 1990; Schroeder et al. 2001). This possibility was in part confirmed by the demonstration that constitutive expression of mammalian type I 5PTase increased drought tolerance of tomato plants (Khodakovskaya et al. 2010). In *Arabidopsis*, multiple *At5PTases* have shown highly responsive to various abiotic stresses including drought (Jia et al. 2019), suggesting that some of *At5PTases* may play roles in response to drought tolerance. To date, however, no plant 5PTase has been reported to enhance drought tolerance.

Altered expression of several 5PTase genes can reduce or increase the level of InsP_3 (Im et al. 2010), resulting in phenotypic changes. In transgenic *Arabidopsis* plants having an increased expression of *At5PTase1* or *At5PTase2* (also known as *AtIP5PII*), induction of ABA/drought-responsive genes by exogenous ABA was reduced compared to that of wild type (Berdy et al. 2001; Sanchez and Chua 2001). Germination of seeds from transgenic *Arabidopsis* plants constitutively expressing *At5PTase2* was insensitive to exogenous ABA (Sanchez and Chua 2001), whereas an *at5ptase2* mutant was hypersensitive to ABA and exhibited increased hypocotyl growth compared to wild type plants grown in the dark (Gunesequera et al. 2007). The functions of *At5PTase6* and *At5PTase7*, also known as cotyledon vascular pattern 2 (CVP2) and CVP2-Like (CVL1), respectively, have been identified to be involved in vascular development based on the finding that mutations in these genes can cause vascular defects (Carland and Nelson 2004, 2009). *At5PTase7* and *At5PTase9* were reported to be involved in the production of reactive oxygen species as well as sensitivity to salt stress. Indeed, *At5ptase7* and *9* mutants exhibit a salt-sensitive phenotype that can be recovered by complementation with wild

type *At5PTase7* and *9* (Golani et al. 2013; Kaye et al. 2011). However, *At5PTase1*, *2*, *3*, and *11* did not show altered salt stress response (Kaye et al. 2011).

Increasing numbers of studies have provided various insights about functions of *Arabidopsis* 5PTases, but very few 5PTases from other plant species have been characterized in detail. In this study, four tomato 5PTases (*Le5PT1-4*) were identified based on amino acid sequence similarity to *Arabidopsis* 5PTases and were examined for responsiveness to abiotic stresses. *Le5PT1* was further examined for its possible role(s) in stress responses and development in tomato by assessing phenotypic changes in *35S:Le5PT1* transgenic plants.

Materials and methods

Plant growth conditions

Seeds from tobacco (*Nicotiana tabacum* 'Wisconsin 38') or tomato (*Solanum lycopersicum* 'Ohio 8245') were surface-sterilized in 95% (v/v) ethanol for one minute, followed by treatment with 50% (v/v) commercial bleach (Clorox® regular bleach containing 5.25% sodium hypochlorite) for 10 min. Sterilized seeds were then rinsed four times with sterile water. The seeds were germinated in Magenta™ boxes (Sigma) containing MS medium (Sigma) supplemented with 2% sucrose, adjusted to pH 5.8, and solidified with 0.7% of plant agar (Sigma). For gene expression studies or transformation, tobacco or tomato seedlings were grown for 3–4 weeks at 23 ± 3 °C under continuous illumination of $50 \mu\text{mol m}^{-2} \text{s}^{-1}$ from fluorescent lamps. For whole plant physiological experiments, 2- to 3-week-old seedlings were transferred into soil-less media (Metro-Mix 360, Scotts-Sierra, Marysville, OH) and grown for 2 months in a greenhouse with a set point temperature of 25 °C and natural photoperiods extended to 16 h using supplemental light from mercury vapor high intensity discharge lamps.

Germination assay was conducted by measuring the time of radicle emergence from tobacco seeds on solid MS media as described above with or without 1.0 μM ABA. ABA stock solution was prepared by dissolving ABA to absolute ethanol. To add ABA, MS media was autoclaved and cooled down to ~60 °C, and then ABA was added to MS media. As for the control MS media, the same volume of absolute ethanol used for 1.0 μM ABA treatment was added. For germination assay, sucrose was excluded from MS media because sugar is closely related to ABA biosynthesis and signaling.

Sequence analysis of tomato EST clones

Tomato EST sequences were identified from a keyword search or a BLAST search of the TIGR tomato database

using At5PTase1 and At5PTase2 amino acid sequences (Berdy et al. 2001; Sanchez and Chua 2001). Four EST clones (cLED2N23, cTOD18D18, cTOC18B10, and cLEX10B15) homologous to At5PTases were obtained from the Clemson University Genomics Institute (Clemson, SC, USA) and confirmed by sequencing analysis using an Applied Biosystems 3730 DNA Analyzer at the Ohio State University Plant-Microbe Genomic facility. Sequence information was used to clone putative tomato inositol polyphosphate 5PTases from 'Ohio 8245' tomato.

5' RACE PCR and cloning of full-length 5PTase cDNAs from tomato

The 5' RACE PCR was performed with total RNA extracted from 1-month-old tomato leaves using an RNeasy plant kit (Qiagen, CA) by following the protocol supplied with the Smart RACE cDNA amplification kit (BD Biosciences Clontech, CA). 5' RACE products were produced using four gene specific primers:

Le5PT1: 5'-GCAAGAAGATTGTCTACGGTACCAAA-3';
 Le5PT2: 5'-AGATCAAAAGCATTCTTCTTCTATATC-3';
 Le5PT3: 5'-AAAGTCAGCATTCCACTCGGCAATT-3';
 Le5PT4: 5'-ATTCCCCTCGCTAAATTGTCTTCCAA-3'.

Each 5' RACE PCR product was cloned into the pGEM[®]-T Easy Vector (Promega, Madison, WI) and sequenced. After confirmation of 5' UTR regions, the coding regions of the four genes were amplified by RT-PCR using the One-Step RNA PCR kit (Takara, Madison, WI, USA) using four gene specific primer sets: forward 5'-ATG CAAATGAAAACAAGAAGG-3' and reverse 5'-AGTACT CTCCAATCTCTAGCA-3' for *Le5PT1*; forward 5'-ATG AGAGAGAGCTCAAGGCAAC-3' and reverse 5'-ACCGCA TCCAACAAGATTCACC-3' for *Le5PT2*; forward 5'-ATG AGGGATGAGTATTCAAAGAG-3' and reverse 5'-AAA AGAAGCATAGTTCGGTATAAC-3' for *Le5PT3*; forward 5'-ATGGCTTTATTTCAAATCATGG-3' and reverse 5'-AGGAATTTTCGGAGGGGACTG-3' for *Le5PT4*. Amplified *Le5PT1* was digested with *SalI/NcoI* and ligated into the corresponding sites of a modified pUC18 vector (Sheen et al. 1995). *Le5PT2-4* cDNA fragments were cloned into the pGEM[®]-T Easy Vector (Promega, Madison, WI).

Abiotic stress treatments of tomato and *Le5PT* expression analysis

For the expression analysis of *Le5PTs*, surface-sterilized tomato seeds were germinated on filter paper in Magenta[™] boxes containing liquid MS medium (Sigma) supplemented

with 1% sucrose and adjusted to pH 5.8. After 1 month, the tomato seedlings were rinsed with distilled water and the medium was replaced with fresh liquid MS medium without sucrose. After one day, the MS medium was replaced with fresh liquid MS medium containing 20% polyethylene glycol (M_w 8000; Sigma), 50 μ M ABA, 200 mM NaCl, or 2% sucrose. For cold and dark treatments, Magenta[™] boxes containing the seedlings were placed in a 4 °C refrigerator or at room temperature without light. Plant samples were harvested at varying times after initiating the stress treatments, frozen in liquid nitrogen, and stored at -70 °C until further analysis.

Total RNA was isolated from tobacco plants using an RNeasy Plant Mini kit (Qiagen, CA, USA). For northern blotting, RNA (10 μ g) was electrophoresed on a 1.2% MOPS/EDTA agarose gel and transferred to a nylon membrane. ³²P-labeled probes were prepared from a plasmid containing *Le5PT1* or *Le5PT2* cDNA by PCR using the following primer sets.

Forward 5'-AGATCATCTCAGGCCTTTTGG-3' and reverse 5'-CATACAAAGCCACTCATCAA-3' for *Le5PT1*; forward 5'-TTACAGTGCTGACCCTGAC-3' and reverse 5'-TTTCGCGAATGATGTCCTC-3' for *Le5PT2*. Gene expression patterns of *Le5PT3* and *Le5PT4* were analyzed by RT-PCR using the One-Step RNA PCR kit (Takara, Madison, WI, USA) and the following primer sets: forward 5'-ATC AACCAGGTGTATGGAG-3' and reverse 5'-CGACGATGA GAGAAGTATG-3' for *Le5PT3*; forward 5'-TGGAGCAGA AGATCCAACAG-3' and reverse 5'-ATGGACATGCTC ACAGAGAC-3' for *Le5PT4*.

Tobacco transformation and expression analysis of tobacco *NtERD10* genes

A 2.2 kb *HindIII/NcoI* fragment (*CaMV 35S:Le5PT1*) from the modified pUC18 vector described above was ligated into the same restriction sites in the pCambia 1302 binary vector to produce *CaMV 35S:Le5PT1-mGFP*, which hereafter is designated as *35S:Le5PT1*. The resulting pCambia 1302 vector was transferred into the *Agrobacterium tumefaciens* strain GV3101 by electroporation. *Agrobacterium*-mediated transformation of tobacco plants was conducted using the leaf disc transformation method (Horsch et al. 1998). Transformants were selected on MS medium containing 50 mg L⁻¹ hygromycin B, transferred to soil-less media, and grown in a greenhouse under growth conditions described above. Homozygous transgenic tobacco lines were screened from transgenic lines with 3:1 ratio of antibiotic (hygromycin B) resistance, and two homozygous *35S:Le5PT1* transgenic tobacco lines were selected for further study.

To examine the salt stress response of transgenic tobacco plants at an early developmental stage, seeds from wild type and transgenic tobacco plants were sterilized and germinated

to grow on MS media containing 0, 50, or 75 mM NaCl but not sucrose for two weeks. Higher salt concentration was not applied because wild type and transgenic tobacco plants did not grow well. The length of cotyledon, hypocotyl, and root were then measured.

To examine the expression of stress-responsive genes in *35S:Le5PT1* transgenic tobacco plants, tobacco seeds were germinated and grown on filter paper floated in liquid MS medium containing 2% sucrose for three weeks. Liquid MS medium was replaced by fresh sucrose-free liquid MS medium with or without 50 μ M ABA or 20% PEG and maintained for one day, and then total RNAs were isolated for RT-PCR analysis. Three stress-responsive genes (*NtERD10B-D*) were examined using the following primer sets: 5'-TTCTTC CGCCTTGTCATCATCCT-3' and 5'-CGACAACCAATT TAGTGCAGGCCA-3' for *NtERD10B*; 5'-GAGGAAAAG CCAACTCATGC-3' and 5'-ACTGGTACAGCCGTGTCC TC-3' for *NtERD10C*; 5'-GAGGACACGGCTGTACCA GT-3' and 5'-GCCAAACAGCAGTAGCACAA-3' for *NtERD10D*.

Plant water usage

Lysimetry method was used to measure and compare daily water use and WUE of 2-month-old wild type and two *35S:Le5PT1* transgenic tobacco lines grown in 2 L pots. Prior to recording daily water loss, six plants from each line were harvested and the leaf area, fresh and dry weight, height, and leaf number were measured for each plant. These data were used as a reference to calculate the WUE. WUE was calculated by dividing the increase in dry weight by the total amount of water lost during a 10-day period. Transpiration and water potentials were calculated as described by Na and Metzger (2017).

Protein extraction from tobacco plants and western blot analysis

To extract protein, leaf tissues (~100 mg) from wild type or transgenic tobacco plants were used as described previously by Na and Metzger (2017). To examine *Le5PT1* expression, western blot analysis was performed by following the method described by Na and Metzger (2017).

Results

Identification of tomato 5PTase genes

Putative tomato inositol polyphosphate 5PTase homologs were identified from tomato expressed sequence tags (ESTs) in the TIGR database. The sequences from EST clones (cLED2N23 for *Le5PT1*; cTOD18D18 for *Le5PT2*;

cTOC18B10 for *Le5PT3*; cLEX10B15 for *Le5PT4*) were used to clone four full-length tomato 5PTase cDNAs, which were designated as *Le5PTs*: *Le5PT1* (accession no. ABV90875.1), *Le5PT2* (ABV90876.1), *Le5PT3* (ABV90877.1), and *Le5PT4* (ABV90878.1). Sequence alignment of the predicted protein sequences of the four *Le5PTs* to six *Arabidopsis* and one human 5PTases revealed the presence of well-conserved catalytic domains (Fig. 1a; Supplementary Fig. S1). Using the BlockMaker program, six conserved regions were found in the four tomato 5PTases, and the organization of these conserved motifs was very similar to those of At5PTases (Fig. 1b). However, human HsType1 5PTase showed slightly different conserved regions compared to the tested plant 5PTases.

Sequence alignment and phylogenetic analysis showed that *Le5PT1* is most similar to At5PTase2, with 51.5% identity and 65.2% similarity. *Le5PT2* showed highest similarity to At5PTase3, with 49.2% identity and 64.4% similarity. *Le5PT3* was more similar to At5PTase6 (CVP2) and 7 (CVL1), which are involved in cotyledon vascular patterning. On the other hand, *Le5PT4* showed a distant relationship with the other three *Le5PTs* and did not group with a specific At5PTase (Fig. 2).

Analysis of tomato *Le5PT* expression

To investigate the spatial and temporal expression patterns of *Le5PTs* in tomato, northern blot and RT-PCR analyses were carried out using total RNAs from tomato seedlings exposed to various abiotic stresses (Fig. 3a) as well as from various tissues (Fig. 3b). *Le5PT1* expression was reduced by dehydration and treatment with 20% PEG. In contrast, *Le5PT1* expression was up-regulated at various time points within 24 h of exposure to 50 μ M ABA and within the first 30 min after treatment with 200 mM NaCl (Fig. 3a). On the other hand, *Le5PT2* and *Le5PT3* expression showed time-dependent increases following initiation of dehydration, PEG, NaCl, and ABA treatments (Fig. 3a). The overall expression patterns of *Le5PT2* and *Le5PT3* were similar under the various stress conditions, indicating that these two genes may be redundant or required for those stress responses. *Le5PT1* expression was up-regulated 1 h after cold stress at 4 °C, but decreased to near control levels after 24 h (Fig. 3a). Since plants were exposed to cold stress in the dark, the amount of *Le5PT1* transcripts at 1 h after cold stress (4 °C) was compared to that of plants grown in the dark at room temperature to determine whether *Le5PT1* expression was influenced by light. The *Le5PT1* transcript levels in plants exposed to cold treatment and grown in the dark at room temperature were similar (Fig. 3a), indicating that *Le5PT1* expression is up-regulated in response to light deprivation rather than cold stress.

Fig. 1 Comparison of amino acid sequences for four putative tomato 5PTases, Le5PT1-4, six *Arabidopsis* 5PTases, and one human Type I 5PTase.

a Alignment of catalytic domains with one known human type I 5PTase, HsTypeI, and six *Arabidopsis* 5PTases, At5PTase1-6. Two conserved catalytic domains were present in Le5PT1-4. **b** Conserved blocks within the 5PTases were generated using Block Maker software in the Blocks WWW Server (https://blocks.fhcr.org/make_blocks.html) and six highly conserved regions were identified. Human TypeI 5PTase did not show the same conserved regions as the other tested plant 5PTases. Colored blocks represent the six conserved regions

A

Gene ID	Accession	Domain I	Domain II
Le5PT1	ABV90875	HDQIFWFGDLNYRISMA...	GEKKRSPAWCDRILWLCK
At5PTase2	AAD10829	HDQVEWFGDLNYRLNMS...	PEKKRAPAWCDRILWLCK
Le5PT4	ABV90878	HDQIFWFGDLNYRLYLE...	GEKQRTPAWCDRILWYCK
Le5PT2	ABV90876	HERITWLGDLNYRINLP...	G...RRNPACDRILSFCK
At5PTase1	AAD10828	HERITWLGDLNYRINLS...	G...KRRPAWCDRIIWNCK
At5PTase3	Q8H0Z6	HERITWLGDLNYRLSSS...	P...KRTPAWCDRVLSYCK
Le5PT3	ABV90877	HDRVILWLGDLNYRIALS...	KDKRRTPAWCDRILWHCG
At5PTase4	Q8GTS0	HDRVILWLGDLNYRIALS...	KAKRRTPAWCDRILWHCS
At5PTase6	Q9LR47	HDRVILWLGDLNYRIALS...	KEKRRTPAWCDRILWFCE
At5PTase5	Q66GQ6	HDRVILWLGDLNYRVALT...	KKKRRTPAWCDRILWKCE
HsTypeI	CAA54676	VKLIFRESNDNRKVMIQ...	YMNTRCPAWCDRIIMSPS

Fig. 2 Phylogenetic analysis of 20 5PTases [15 from *Arabidopsis* (At5PTases), four from tomato (Le5PT1-4), one from human (HsTypeI)]. Phylogenetic trees were generated using UPGMA embedded in MEGA7 software (Kumar et al. 2016). The numbers indicate the percentage of replicate trees with 1000 bootstrap replications. Accession numbers used for At5PTase7~15 are: NP_180761, NP_181280, NP_001323555, NP_001078529, NP_175182, OAP09396, XP_020867570, NP_001189651, and NP_176736. The remaining 5PTase accession numbers are shown in Fig. 1a

To examine the expression patterns of *Le5PTs* in different tissues, RT-PCR analysis was performed using total RNAs from old and young leaves, stems, and flowers taken from 3-month-old tomato plants grown in a greenhouse. *Le5PT1* transcript levels were significantly higher in old leaves, stems, and flowers compared to young leaves, which had nearly undetectable transcript levels. The amount of *Le5PT4* transcripts was very high in both old and young leaves compared to stems and flowers (Fig. 3b).

Ectopic expression of *Le5PT1* in tobacco plant

InsP₃ levels in plants increase under environmental stresses, and are likely regulated, in part, by either reduced biosynthesis or reduced activity of InsP₃-specific 5PTase. Among the four *Le5PT* genes isolated, only *Le5PT1* was down-regulated by abiotic stresses (Fig. 3a), indicating that *Le5PT1* may drive elevations in InsP₃ levels under adverse environmental conditions and that altered *Le5PT1* expression may affect abiotic stress responses or promote phenotypic changes. Thus, *Le5PT1* was selected to study its role in response to abiotic stresses and development. Although evaluation of *Le5PT1* function in tomato plants would be ideal, it was difficult to generate *35S:Le5PT1* transgenic tomato lines with suitable transgene expression. In this study, therefore, *35S:Le5PT1* transgenic tobacco plants were generated and used. Two transgenic tobacco lines (NS11 and NS 30) expressing *Le5PT1* were chosen for further characterization

Fig. 3 Expression patterns of the four putative tomato *Le5PTs*. **a** Northern blot (*Le5PT1* and *Le5PT2*) or RT-PCR analysis (*Le5PT3* and *Le5PT4*) were conducted using total RNAs isolated at the indicated time points (0, 0.5, 1, 3, and 24 h) from 1-month-old tomato seedlings exposed to dehydration, 50 μ M ABA, 200 mM NaCl, 20%

PEG, darkness at room temperature, or 4 °C. **b** Expression patterns of the four *Le5PTs* in different tissues. RT-PCR analysis was conducted using total RNAs extracted from old leaves (OL), young leaves (YL), stems (S), and flowers (F) from 3-month old plants grown in a greenhouse

Fig. 4 Construct used for tobacco transformation and analysis of transgene expression. The tomato *Le5PT1* gene was ligated between the strong tobacco mosaic virus *CaMV* 35S promoter and mGFP in the pCambia 1304 binary vector. Transcript levels of *Le5PT1* in 1-month-old wild type (WT) and two homozygous transgenic tobacco lines (NS11, NS30) were examined by RT-PCR and western blot. The expression of *Le5PT1-mGFP* fusion proteins was detected using an anti-GFP rabbit polyclonal antibody

(Fig. 4). Transgene expression in these two lines was validated by western blot analysis, and the NS30 line showed higher expression of fusion proteins (Fig. 4).

35S:*Le5PT1* tobacco plants show retarded growth

The *Arabidopsis* mutants, *at5ptase1* and *at5ptase2*, exhibited an increased hypocotyl growth, which is coincident with elevated InsP_3 levels (Gunasekera et al. 2007). If *Le5PT1* is homologous to *At5PTase2*, then transgenic plants expressing *Le5PT1* might show an altered phenotype in seedling growth. Hypocotyl growth of 35S:*Le5PT1* seedlings was slightly reduced compared to that of wild type, although the difference was not significant (Fig. 5a, b). However, the transgenic seedlings did exhibit altered growth in cotyledons, indicating that *Le5PT1* affects plant development.

In a previous study, InsP_3 and its precursor $\text{PtdIns } 4,5$ bisphosphate accumulated following exposure to salt stress (DeWald et al. 2001), suggesting that altered expression of 5PTase may affect salt stress response by altering InsP_3 levels. To examine whether salt stress affects the development of 35S:*Le5PT1* seedlings, cotyledon, hypocotyl, and root length of 14-day-old seedlings grown in MS medium containing 0, 50, or 75 mM NaCl were measured. Hypocotyl growth was not affected by NaCl treatment in either transgenic or wild type plants (Fig. 5b). In addition, the cotyledon length of 35S:*Le5PT1* transgenic seedlings did not significantly

Fig. 5 Salt stress response of *35S:Le5PT1* seedlings. **a** Two-week-old *35S:Le5PT1* transgenic tobacco seedlings grown in MS media with or without 50 mM NaCl. **b** Hypocotyl length, **c** cotyledon length, and **d** root length of seedlings grown in 0, 50, or 75 mM NaCl. Vertical bars

denote standard deviations of the mean ($n=15$). Different letters indicate significant differences among the three genotypes ($P<0.05$) in a Student–Newman–Keuls pairwise multiple comparison

differ from wild type following salt stress, but the NS30 and NS11 transgenic lines had lower linear regression than wild type (Fig. 5c), indicating that cotyledon growth of *35S:Le5PT1* seedlings was less sensitive to salt stress (WT: $y = -0.5667x + 4.6889$; NS11: $y = -0.45x + 3.7667$; NS30: $y = -0.2167x + 2.6222$; $R^2 > 0.98$). In the absence of salt, root growth of *35S:Le5PT1* and wild type seedlings was similar, but following NaCl treatment the root growth of transgenic plants was significantly decreased relative to wild type (Fig. 5d), indicating that roots of *35S:Le5PT1* plants were more sensitive to salt stress than wild type.

To examine whether *35S:Le5PT1* plants also exhibit retarded growth at the late developmental stage, the growth parameters of 3-month-old tobacco plants were analyzed. Transgenic plants exhibited significantly reduced growth compared to wild type plants for all measured parameters, including height, dry weight of entire shoots, and leaf area (Fig. 6; Supplementary Table S1), indicating that *Le5PT1* expression might affect plant growth during the entire developmental period.

It was reported that disruption of *At5PTase2* gene expression altered ABA sensitivity of seed in germination (Gunasekera et al. 2007; Sanchez and Chua 2001). To examine whether *Le5PT1* is also involved in altered ABA response in seed germination as does *At5PTase2*, we carried out the seed germination assays using seeds from *35S:Le5PT1* transgenic plants and found that the germination rate of *35S:Le5PT1* seeds and wild type seeds in response to exogenous ABA did not differ significantly (Supplementary Fig. S2), indicating that *Le5PT1* may be not involved in ABA signaling related to seed germination.

Effect of *Le5PT1* expression on water use efficiency (WUE) in tobacco plants

At5PTase1 and *At5PTase2* are known to be involved in ABA signaling (Jia et al. 2019), and it is well known that ABA is tightly correlated to water regulation in plants. Therefore, we examined whether water regulation of *35S:Le5PT1* transgenic plants was affected by changes in stomatal activity by

Fig. 6 The effects of ectopic *Le5PT1* expression on tobacco plant growth. **a** Three-month-old plants grown in a greenhouse. **b** Plant height (cm). **c** Dry weight of the entire shoot (g). **d** Leaf area (cm²).

Vertical bars denote standard deviations ($n=12$). Different letters above the columns denote significant differences among the three genotypes ($P<0.05$) in a Tukey pairwise multiple comparison

measuring various growth parameters of *35S:Le5PT1* transgenic plants. Wild type and *35S:Le5PT1* transgenic lines showed no significant difference in daily whole plant water loss and transpiration per unit leaf area (Fig. 7a, b). Measurement of water potential indicated that transgene expression had no overall effect on plant water status (Fig. 7c), indicating that *Le5PT1* expression had only a limited effect on water status. On the other hand, *35S:Le5PT1* transgenic plants exhibited a lower WUE compared to wild type (Fig. 7d), suggesting that WUE is reduced because growth rates are reduced more in the transgenic plants than water use does. These results suggest that *Le5PT1* may not be involved in ABA-mediated stomatal closure.

Downregulation of *LEA* gene expression in *35S:Le5PT1* tobacco plants

To examine whether *Le5PT1* overexpression affects the levels of three *Late Embryogenesis Abundant (LEA)* group 2 genes (*NtERD10B*, *C*, and *D*) in tobacco, RT-PCR analysis was conducted using total RNAs from wild type and transgenic tobacco seedlings treated with 20% PEG (to mimic

dehydration) or 50 μ M ABA for one day at room temperature under continuous light. The level of *NtERD10B* transcripts was lower in transgenic plants than wild type plants treated with 20% PEG, but the transcript levels were similar between *35S:Le5PT1* and wild type plants treated with ABA (Fig. 8), indicating that *Le5PT1* may be involved in water stress responses in an ABA-independent manner. The expression levels of both *NtERD10C* and *D* were also very similar under both conditions.

Discussion

Arabidopsis inositol polyphosphate 5PTases regulate levels of various phosphoinositides, and thus play key roles in several developmental processes and abiotic stresses, such as salt stress (Golani et al. 2013; Kaye et al. 2011), plant response to ABA and ABA-induced stomatal activity (Burnette et al. 2003), cotyledon vascular patterning (Carland and Nelson 2004), secondary cell wall synthesis (Zhong et al. 2004), seedling growth (Gunasekera et al. 2007), pollen dormancy (Wang et al. 2012), and gravitropic growth

Fig. 7 Water usage in *35S:Le5PT1* tobacco plants. **a** Daily water loss. **b** Cumulative water loss. **c** WUE was lower in transgenic plants than WT. **d** Water potential was not changed in *35S:Le5PT1* plants. Error

bars denote standard deviations ($n=12$). Different letters above the columns denote significant differences among the three genotypes ($P < 0.05$) in a Tukey pairwise multiple comparison

Fig. 8 Down-regulation of drought-inducible *NtERD10B* transcript levels in *35S:Le5PT1* transgenic plants. The expression of three *LEA* group 2 genes, *NtERD10B*, *C*, and *D*, was analyzed by RT-PCR using RNAs extracted from transgenic tobacco seedlings treated with 50 μ M ABA or 20% PEG for 24 h

(Salinas-Mondragon et al. 2010). Despite these crucial roles, to our knowledge no other plant 5PTases have been extensively characterized with the exception of *Arabidopsis* At5PTases. Here, four putative inositol polyphosphate 5PTase homologs, Le5PT1-4, were identified from *S. lycopersicum* 'Ohio 8245' and physiological role of Le5PT1 was investigated.

Phylogenetic analysis based on predicted amino acid sequences revealed close relationships between Le5PT1 and *Arabidopsis* At5PTase2, Le5PT2 and At5PTase3, and

Le5PT3 with both At5PTase6 and At5PTase7 (Fig. 2). In *Arabidopsis*, transgenic plants expressing elevated levels of At5PTase2 transcripts exhibited ABA insensitivity during seed germination (Sanchez and Chua 2001). Meanwhile, *5ptase1* and *5ptase2* mutants were hypersensitive to ABA and showed higher levels of *InsP₃* compared to wild type plants (Gunesequera et al. 2007). Interestingly, *Arabidopsis* *5ptase1* and *5ptase2* mutants contained higher amounts of endogenous ABA than wild type plants (Gunesequera et al. 2007), suggesting that 5PTases may be involved in ABA

stability or biosynthesis. This potential role could explain why seeds of plants constitutively expressing *At5PTase1* and 2 were hypersensitive to ABA in seed germination. The amino acid sequence similarity between *Le5PT1* and *At5PTase2* suggested that seeds from *35S:Le5PT1* transgenic plants could be similarly insensitive to exogenous ABA in germination, yet no significant difference in the seed germination frequencies was seen between wild type and *35S:Le5PT1* transgenic tobacco lines following application of exogenous ABA (Supplementary Fig. S2). This result suggests that *Le5PT1* likely is not involved in ABA signaling in seed germination. Despite the apparent functional differences between *At5PTase2* and *Le5PT1*, these genes showed similar transcription patterns in response to salt stress and low temperature as shown in Fig. 3a and results reported by Golani et al. (2013). However, *Le5PT1* expression was downregulated by water deficit (Fig. 3a), whereas *At5PTase2* showed slight upregulation (Golani et al. 2013).

The *35S:Le5PT1* tobacco plants exhibited reduced growth, and the degree of reduction correlated with *Le5PT1*-mGFP protein levels (Fig. 4). The reduction in growth, including that seen for leaves and stems, suggests that *Le5PT1* may play a regulatory role during leaf expansion and stem growth. The cotyledon and the hypocotyl length of *35S:Le5PT1* seedlings were also reduced compared to that of wild type (Fig. 5), indicating that increased levels of *Le5PT1* transcripts may reduce growth of tobacco plants. This result is supported by the finding that the *Arabidopsis 5ptase1* mutant exhibits increased hypocotyl growth. Moreover, inositol polyphosphate 5PTase was recently shown to be involved in internode growth in maize (Avila et al. 2016). In maize, the putative inositol polyphosphate 5PTase *Brevis plant1* (BV1) is involved in growth through regulation of internode elongation. Indeed, *bv1* mutants exhibited stunted growth due to reduced internode elongation (Avila et al. 2016), which is in contrast to the effects of *5ptase1* and *5ptase2* mutants on *Arabidopsis* growth. The stunted phenotype of *bv1* plants is similar to that seen in *35S:Le5PT1* tobacco plants (Fig. 6a). Taken together, these findings suggest not only that the role of 5PTases in development may differ among plant species, but also that 5PTases are required for normal plant growth.

Altered levels of *At5PTase1* or *At5PTase2* transcripts affect expression of stress-responsive genes in *Arabidopsis* (Berdy et al. 2001; Sanchez and Chua 2001). Similarly, ectopic expression of *Le5PT1* reduced the expression level of a stress-responsive gene, *NtERD10B*, following PEG treatment (dehydration condition) of tobacco plants, suggesting that *Le5PT1* may be involved in water stress response. Hydrophilic group 2 LEA proteins accumulate in vegetative tissues in response to several environmental stresses including water deficit, salt, and cold stress (Hinniger et al. 2006). LEA proteins also promote stress tolerance by reducing

water loss under these adverse conditions (Hinniger et al. 2006). In tobacco, expression of four group 2 LEA genes, *NtERD10A-D*, was highly induced by water deficit and low temperature, with the exception of *NtERD10A*, which had low expression levels at low temperature, and they were highly induced in transgenic plants overexpressing the dehydration-responsive element (DRE)-binding protein 1A, *DREB1A* (Kasuga et al. 2004). The finding that *35S:Le5PT1* tobacco plants showed reduced levels of *NtERD10B* transcripts in response to water deficit indicates that *Le5PT1* may have negative effects on drought response by inhibiting transcriptional regulation of DRE binding proteins in tobacco plants. This reduction may reduce the timely ability of *35S:Le5PT1* plants to adapt to abiotic stress, resulting in a reduction in plant growth and WUE (Fig. 7c).

In summary, tomato *Le5PT1* has high similarity to *Arabidopsis At5PTase2* and the reduction in growth seen for transgenic plants with ectopic expression of *Le5PT1* suggests that *Le5PT1* plays a role in plant growth. Meanwhile, *Le5PT1* did not change ABA sensitivity in germination tests, in contrast to the enhanced sensitivity conferred by *Arabidopsis At5PTase2*. The transcript levels of drought-inducible *NtERD10B* were decreased in *35S:Le5PT1* tobacco plants in response to water deficit but not exogenous ABA, suggesting that *Le5PT1* may be involved in the water stress response in an ABA-independent manner.

Acknowledgements This research was supported by funds from the Kiplinger Endowment for Floriculture to JDM and by a SEEDS grant for graduate research to JKN from the Ohio Agricultural Research and Development Center.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interests.

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