EXPRESSION PATTERNS OF PHOSPHATE TRANSPORTER GENE *EcgPT* ASSOCIATED WITH THE SALT STRESS RESPONSE IN PERENNIAL EUCALYPT TREE HYBRID CLONES

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ABSTRACT

Salinity effects on plant can often be related to mineral ion content alteration, including phosphate. Under saline conditions, phosphate levels were reported to decrease in plants. Such effects could indirectly affect intracellular phosphate levels, leading to phosphate deficiency, which in turn leads to increased activities of phosphate uptake mechanisms. This research was aimed to investigate the effects of salinity on the expression changes of phosphate transporter genes isolated from *E. camaldulensis* x *E. globulus* hybrid clones subjected to salt concentrations of 0 (control), 50, 100, and 150 mMNaCl. Fragments of genes (1164bp long and encodes 304 amino acid polypeptides) known to be involved in phosphate uptake were identified and isolated by RACE from these hybrids, encoding a Phosphate Transporter (*EcgPT*). Expression studies using Northern-Blot analysis revealed that the expression of *EcgPT* was found to be affected by salt, suggesting a direct effect of salinity on phosphate uptake. *Ecg*PT was expressed differently in different clones, indicating different degrees of phosphate transporter activation in order to tolerate salt stress. Molecular data are discussed in relation to measurements of ion levels in different organs of different clones and under various salt regimes during the course of the hydroponic experiment.

Keywords: Phosphate transporter gene, salinity stress, phosphate content, eucalypt

I. INTRODUCTION

Plants have developed a variety of mechanisms to survive environmental stress. A dual-mechanism model for ion uptake, including phosphate, has been proposed for many plants (Chrispeels *et al.,* 1999; Epstein, 1966). This is characterized by a low-affinity uptake system operating at high (mM) concentrations, and a high-affinity system functioning at low (M) concentration of ions. A high-affinity inorganic phosphate transporter (PT) was first recognized in *Saccharomyces cerevisiae* as the *PH084* gene (Bun-Ya *et al.,* 1991). Later, several high-affinity Phosphate transporters have been identified and isolated in *Arabidopsis,* all belonging to the same gene family, *Pht1* (Muchhal *et al.,* 1996; Smith *et al.,* 1997). Homologues to PT gene family have also been isolated from potato (Leggewie *et al.,* 1997), tomato (Daram *et al.,* 1998; Liu *et al.,* 1998), and barley (Rae *et al.,* 2003). The sizes of PT mRNAs differ in different species, but lengths of their Open Reading Frames (ORF) are similar. This high similarity of ORF among various plants indicates similar function of PT gene in those plant species, while the various lengths of untranslated regions in different species might reflect the host plant specific differences in PT gene function (Lam and Maisel, 1999).

High-affinity phosphate uptake system in all plants respond to phosphate deprivation with a dramatic increase in transcript levels (Schachtman *et al.,* 1998). Transcriptional regulation of *PT*genes primarily responded to the P status of the whole plant, and its protein level was reversible upon resupply of Pi (Liu *et al.,* 1998; Muchhal and Raghothama, 1999).

Salinity is believed to disrupt the mechanisms that control intracellular orthophosphate (Pi)

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concentrations (Ashraf and Ahmad, 2000), and mechanism of general transporter activities (Chen *et al.,* 2012). Such effects could indirectly regulate intracellular P levels, and lead to phosphate deficiency. This led to the hypothesis that phosphate deficiency might be a secondary effect of salinity (Plaxton and Carswell, 1999), and that salt-stressed plants may actively regulate their P uptake and transport systems to maintain uptake and distribution of P.

Some eucalypt species are known for their tolerance to salt, but little is understood on how salt stress might affect nutrient uptake mechanism such as phosphate transporter. Therefore, the study aimed to investigate whether *EcgPT* (*Eucalyptus camaldulensis* x *globulus Phosphate Transporter*) expression is 1) regulated by salt, indicating an effect of salt stress on P uptake, and 2) differentially regulated in different clones, indicating various salt tolerance mechanisms.

II. METHODOLOGY

A. Plant Material and Growing Conditions

Six-month-old *E. camaldulensis* x *E. globulus* hybrid cuttings purchased from Saltgrow Company (from 9 clones coded as clones 1-9) were grown in hydroponic tubs in the glasshouse at a temperature of $21^{\circ}C/18^{\circ}C$, under 14h/day and 10h/night regime. After growing in half-strength nutrient solution for the first week the plants were transferred to full strength nutrient solution (Morris, 1984). In the third week, salt in the form of NaCl was added to the full-strength nutrient solution to final concentrations of 0mM (as control), 50mM, 100mM, and 150mM. Nutrient and salt solutions were renewed with fresh solution every week for the five-week duration of the experiment, and plants were harvested every week from different clones and different salt concentrations.

Upon harvesting, plants were divided into 3 parts: leaves, stems, branches, and roots. Some young leaves and parts of the root material were taken for nutrient analysis. The remaining samples (leaves, stems, roots) were separated into 50 mL Falcon tubes, immediately snap-frozen in liquid nitrogen and stored at $-80^{\circ}C$ for total RNA extraction.

B. 5' and 3' RACE (Rapid Amplification of cDNA Ends)

Messenger RNA (mRNA) used for RACE was isolated from total RNA using a Promega mRNA isolation kit. The synthesized single stranded cDNA reactions produced were used as templates for amplification of 5' and 3' ends using the Clontech SMART™ RACE cDNA Amplification Kit.

To amplify *PT* cDNA fragments, two GC-rich Gene Specific Primers (PF1: CTCGTTACACCGCCC-TAGTGGCCAAGA and PR1: AGCGACCGTGAACCAG-TATCCAGGGAC) were designed based on the sequence of previously amplified *PT* DNA PCR products (data not shown) to amplify the gene's 5' and 3' ends through RT-PCR. RACE reactions were carried out using *Taq* polymerase (Fermentas) and the following cycle profile: initial denaturation of cDNA at $95^{\circ}C$ for 20sec, 35-38 cycles of amplification by denaturing at $95^{\circ}C$ for 5sec, annealing at $60^{\circ}C$ for 1min and extending at $70^{\circ}C$ for 2min, and a final extension at $70^{\circ}C$ for 4 min.

C. Sequencing and Sequence Analysis

Sub-cloned cDNAs were labeled using the CEQ 2000 Dye Terminator Cycle Sequencing Kit using T3 (5'-AATTAACCCTCACTAAAGGG-3') or T7 (5'-GTAATACGACTCACTATAGG-GC-3') primers. Sequences were determined using a CEQ8000 DNA Sequencer (Beckman Coulter). Sequences were compared with existing publicly available sequences using BLAST (http://www.-ncbi.nlm.nih.gov). Sequence alignment and analysis were performed through facilities available at Bionavigator (http://www.biona-vigator.com), and the Expasy Molecular Biology (http://kr.expasy.org), as well as additional sequence analysis such as PSORT analyses.

d. Northern Blot Analysis

Analysis was conducted as prescribed by

Amersham pharmacia in 2004. Probes designed to 3' end used for Northern Blotting was *EcgPT* DNA fragments (data not shown). Amounts of RNA loaded onto each gel were visualized and compared using Ethidium Bromide (EtBr) staining. DNA probes were radioactively labeled using the Decalabel™ DNA labeling kit (Fermentas) and [α-³²P]-dATP (Perkin Elmer). Labeling, prehybridization and hybridization methods were done following manufacturer's instructions.

III. RESULT AND DISCUSSION

a. *EcgPT* Race

The cloned *EcgPT* (*EcgPT*-3') was 1096bp in length, and combination with a 68bp sequence from the genomic *PT* DNA fragment (located 5' direction) resulted in a 1164bp sequence encoding a 305 amino acid polypeptide. This fragment contained a 3'-untranslated region of 249bp length. Alignment to other PTs at the deduced amino acid level confirmed the end of the coding region and the position of *EcgPT* in relation to other *PTs* (Figure 1).
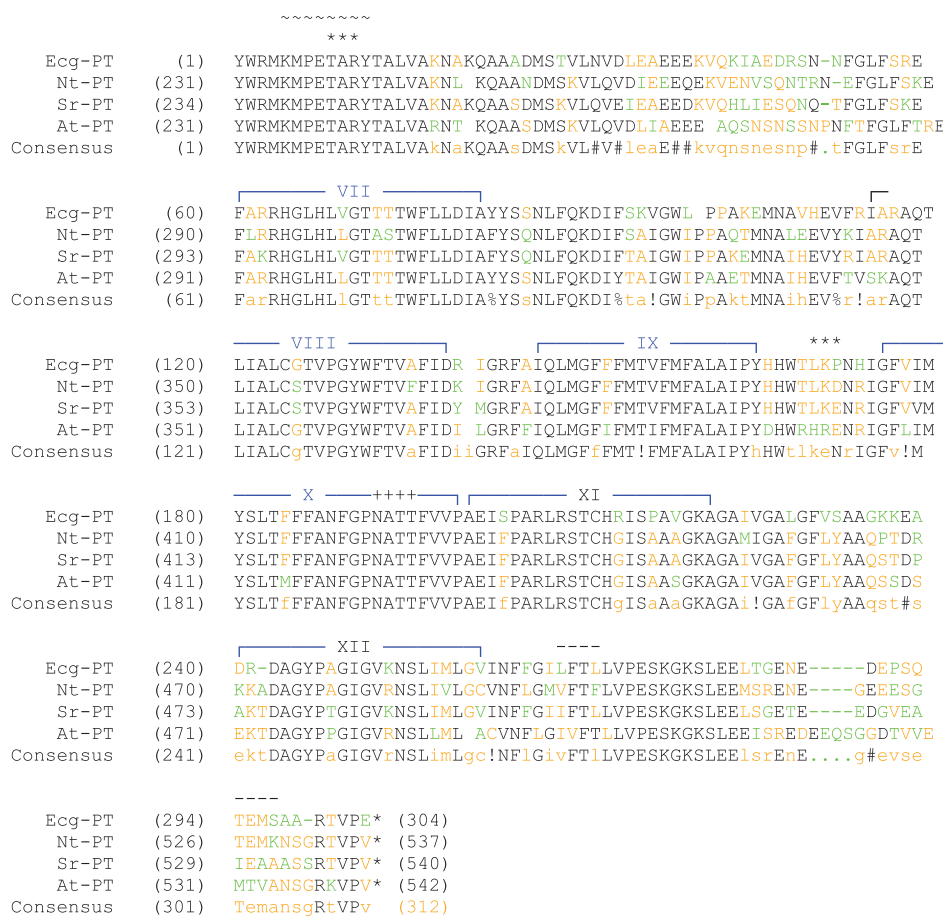

Figure 1. Deduced amino acid alignment of *EcgPT* and three known *PTs*: *Nicotiana tabacum* (NtPT, acc. no: AAF74025), *Sesbania rostrata* (SrPT, acc. no: CAC28219), and *Arabidopsis thaliana* (AtPT, acc. no: BAA24280). Amino acids conserved among all four clones (full consensus) are shown in black, upper-case letters; medium consensus (those conserved among three or two clones) are represented by orange, lower-case letters. Other consensus symbols are: ! (I and V), $ (L and M), % (F and Y), and # (N, D, Q, E, B, and Z). Asterisks indicate the stop codon. Possible transmembrane domains are indicated by Roman numerals (generated based on hydropathy analysis (http://saier-144-37.ucsd.edu) and *PT* gene structure in tomato (Daram *et al.* 1998) and *Arabidopsis* (Muchhal *et al.* 1996)). Consensus sites for phosphorylation by protein kinase C (***), site for phosphorylation by casein kinase II (----), site for N-glycosylation (+++), and site for tyrosine phosphorylation (~~~~~) are also shown.

Table 1. Nucleotide and amino acid sequence identity[*] between available Pts[**]

	Ecg-PT	Sr-PT	Nt-PT	At-PT	St-PT	Os-PT
Ecg-PT	---	80.8 (88.3)	76.1 (86.4)	71.9 (82.7)	70.1 (79.4)	68.5 (80.5)
Sr-PT	73.1	---	80.1 (89.3)	76.9 (86.2)	76.5 (84.7)	74.6 (84.0)
Nt-PT	69.1	71.3	---	74.8 (86.0)	76.5 (85.7)	74.0 (84.1)
At-PT	67.7	68.3	68.0	---	67.9 (79.2)	66.0 (77.3)
St-PT	67.6	69.7	70.1	65.8	---	68.5 (80.1)
Os-PT	67.8	65.8	62.7	66.3	61.2	---

[*] left-hand side of the diagonal matrix = nucleotide identity, right-hand side of the diagonal matrix = amino acid identity (similarity shown in brackets)

[**] Percentages of identity and similarity shown were determined by alignment of two sequences at a time (www.ebi.ac.uk). Accession numbers of nucleotide and protein sequences of each clones are as follows:

Sr-PT (*Sesbaniarostrata*, nucleotides: AJ286744, protein: CAC28219),

Nt-PT (*Nicotianatabacum*, nucleotides: AF156696, protein AAF74025),

At-PT (*Arabidopsis thaliana*, nucleotides: NM_128843, protein: BAA24280),

St-PT (*Solanumtuberosum*, nucleotides: X98891, protein: CAA67396),

Os-PT (*Oryza sativa*, nucleotides: AF536968, protein: AAN39049).

Based on its deduced amino acid sequence, a PSORT analysis (Prediction of Protein Localization Sites, http://psort.nibb.ac.jp) predicted that *EcgPT* is likely to be localized in mitochondrial inner membranes (certainty= 0.746) or in the plasma membrane (certainty= 0.600). This result suggests that *EcgPT* encodes a transmembrane PT (refer to Figure 1). When the nucleotide sequence of the *EcgPT* fragment was aligned to cDNAs of potato and *Arabidopsis thaliana* PTs, it showed 67.6% and 67.7% identity, respectively. A summary of identity values among available PTs is shown in Table 1.

b. *EcgPT* Expression Patterns in *E. camaldulensis* x *E. globulus* Hybrid Clones

Effects of salinity on the expression of Phosphate transporter genes in *E. camaldulensis x E. globulus* hybrids (*EcgPT*) were studied by subjecting various clones to different salt concentrations (0mM, 50mM, 100mM, and 150mM). When membranes containing total RNA were probed with *EcgPT* (DNA), $\pm$ 2.9 kb transcripts were detected (Figure 2). After two weeks of salt stress, *EcgPT* expressions in the leaves of clone 7 were not noticeable at 0 and 50 mM NaCl, slightly increased at 100 mM NaCl and reached its peak at 150 mM NaCl concentration (Figure 2A). Similar patterns were noticed for stem tissues of clone 5 exposed to salt for three weeks (Figure 2B). In this clone, however, expression at salt levels 100mM and 150mM was higher than that found for leaves of clone 7. The initial conclusion from these

observations is that the expression of *EcgPT* is regulated by salt.

In order to examine whether *EcgPT* gene expression in various organs was affected differently by increasing salt concentrations, total RNA was isolated from roots, stems and leaves after plants were exposed to various salt levels. In clone 9 (Figure 3), *EcgPT* transcripts in leaves were clearly detectable under control conditions, and gradually increased with increasing salt levels, reaching a peak at 100mM salt level, but declined slightly at salt level. In roots, *EcgPT* transcripts were noticeable even without salt treatment. Expression slightly increased when plants were treated with 50mM NaCl. At 100mM NaCl expression declined again, and remained constant at the highest salt level. *EcgPT* expression in stems was not noticeable in plants not subjected to salt treatment or at 50mM NaCl. Increasing salt concentration to 100mM slightly increased *EcgPT* transcript levels, but at 150mM NaCl *EcgPT* expression could not be observed.

EcgPT was differentially expressed not only in plant organs, but significant differences also were observed between different clones. *Ecg*PT transcripts as they were accumulated in leaves of all clones (clones 1-9) exposed to 150mM NaCl for two weeks are shown in Figure 4A, illustrating different activity levels of *EcgPT* genes in different clones in response to the same salt stress levels. Accumulation of *EcgPT* transcripts could be related to the accumulation of P in leaves of the corresponding groups of clones (Figure 4B), i.e.
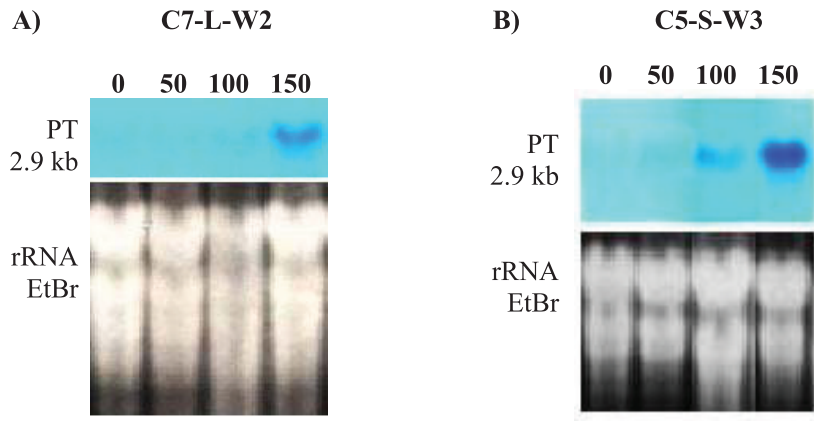
Figure 2. *EcgPT* expression in *E. camaldulensis x E. globulus* as induced by NaCl. **A**. Leaves (L) of clone 7 (C7) after two weeks (W2) of exposure, and **B**. Stems (S) of clone 5 (C5) after three weeks (W3) of exposure to 0mM, 50mM, 100mM, and 150mM NaCl. Total RNA loaded in each lane was 20μg.
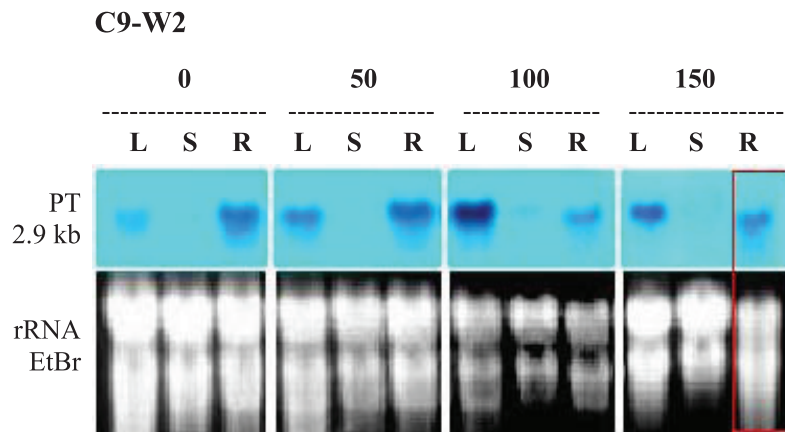

Figure 3. *EcgPT* expression in different plant organs (L = leaves, S = stems, R = roots) of *E. camaldulensis x E. globulus* clones. Clone 9 after two weeks of exposure (W2) to 0mM, 50mM, 100mM, and 150mM NaCl. Total RNA loaded for each sample was 20μg. EtBr-staining confirmed that equal amounts were loaded in each lane. Boxed lanes are suspected to contain degraded RNA.
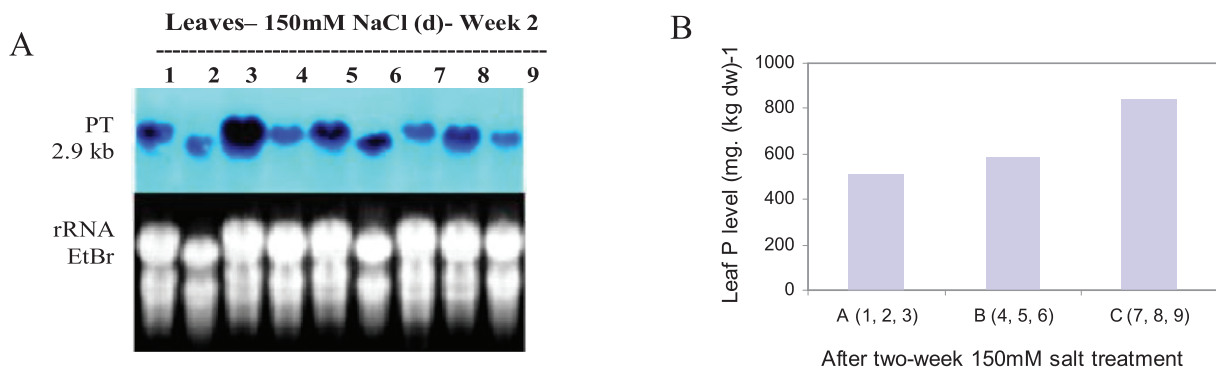

Figure 4. *EcgPT* expression and P levels in plant leaves. A) Expression in different *E. camaldulensis x E. globulus* clones after different lengths of exposure to 150mM NaCl. Total RNA loaded in each lane was 20μg. B) Leaf P (mg. (kg dw)$^{-1}$) for corresponding clones (group A for clones 1, 2, 3; B for 4, 5, 6; and C for 7, 8, 9).

groups with lower leaf P content showed higher *EcgPT* expression levels.

c. Amplification, Cloning and Sequence Analysis of *EcgPT*

A Phosphate Transporter (PT) cDNA from *E. camaldulensis x E. globulus* was amplified by RACE, and further characterized and used for gene expression studies. A composite cDNA was assembled from two overlapping clones, *EcgPT*-3', amplified by RACE, and *EcgPT* DNA, a cloned PCR product amplified from genomic DNA. The *EcgPT* is 1096bp long and encodes a 305 amino acid polypeptide. *EcgPT* also contains a 3'-untranslated region of 249bp and a stop codon (TAA) located at the 913th nucleotide (Figure 1). RACE and the subsequent cloning of PTs from *E. camaldulensis x E. globulus* only yielded a single *EcgPT* clone. However, it is common for some plant species to have several PTs, as is the case for *Arabidopsis thaliana* with *AtPT1* and *AtPT2* (Muchhal *et al.,* 1996), or potato with *StPT1* and *StPT2* (Leggewie *et al.,* 1997).

Alignment results showed high homology between *EcgPT* and high-affinity PTs from a diverse range of plant species, and indicated that the *EcgPT* gene fragment is not located towards the 5' end of the gene-coding region when compared to other PTs (Figure 1). Being incomplete, *EcgPT* might not contain consensus sequences specific to other PT genes. As an example, *EcgPT* does not contain another site for phosphorylation by protein kinase, which is located around transmembrane domains IV. As expected, the localization of *EcgPT* was predicted by PSORT to be within the plasma membrane (certainty= 0.600), although PSORT also predicted it to be localized in mitochondrial inner membrane (certainty=0.746), Golgi body (certainty=0.400), and chloroplast thylakoid membrane (certainty=0.349). These results underline the function of *EcgPT* as a transporter/symporter.

d. Expression Studies of *EcgPT*

P deprivation leads to an increase in transcript levels of Phosphate transporters, as demonstrated in plants of Arabidopsis (Smith *et al.,* 1997), tomato (Mutikara *et al.,* 2001), potato (Leggewie *et al.,* 1997), barley (Rae *et al.,* 2003) and green algae

Dunaliellasalina (Li *et al.,* 2012). In this study, exposure to NaCl increased the accumulation of *EcgPT* transcripts in *E. camaldulensis* x *E. globulus*, resembling symptoms of plants deprived of P (Figure 2). In fact, at higher salt concentrations, accumulation of *EcgPT* transcripts was observed to increase in all plant organs, indicating that increasing NaCl levels further reduced P uptake and hence P levels in plants. In addition, the expression of *EcgPT* in response to salt treatment differed in an organ-specific manner (Figure 3).

In plants grown without stress, *EcgPT* expression was noticeable in roots and leaves, but not in stems. These results are consistent with findings in potato, where StPT1, a high-affinity PT, was highly expressed in roots, source leaves and flower buds, but not in stems (Leggewie *et al.,* 1997). This ubiquitous and relatively high base level of expression in leaves and roots might indicate that *EcgPT* has important physiological functions in *E. camaldulensis* x *E. globulus* even in the absence of stress. Roots are the organs involved in nutrient acquisition, and once roots take up phosphate, this will be transported in the xylem to the leaves, and hence expression patterns of *EcgPT* genes in roots and leaves correlated well with their physiological function.

NaCl-induced *EcgPT* expression differed between clones (Figure 4A). Exposure to 150mM NaCl for two weeks increased *EcgPT* expression in leaves however, some exceptions were noted. Clones 2, 7 and 9 for example showed lower expression than other clones. When comparing different expression levels of *EcgPT* to accumulated P levels in corresponding clone groups (Figure 4B), it seems that despite relatively lower expression levels after longer exposure to stress, higher *EcgPT* expression levels initially might be associated with lower P accumulation in leaves. For example, clones 7, 8, and 9 (Group C), whose leaf P levels were the highest on average showed the lowest *EcgPT* expression levels. Consistently, Group A showing the lowest P accumulation levels responded with higher *EcgPT* expression on average.

IV. CONCLUSION

Although it is difficult to answer with certainty

whether different *EcgPT* expression levels are in fact related to leaf P levels, and whether these are largely affected by salt stress, it can be concluded that *EcgPT* expression in *E. camaldulensis* x *E. globulus* clone hybrids was regulated by salt levels in organ-specific manner and differently regulated in different clones. More research is needed to clearly establish the connection between *EcgPT* activation and plant physiological state (e.g. nutrient status) and to understand how *EcgPT* gene expression is influenced by environmental stresses.

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