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Expression pattern and subcellular localization of Arabidopsis purple acid phosphatase AtPAP9

Katayoun Zamani [a], Tahmineh Lohrasebi [a], Mohammad S. Sabet [b], Mohammad A. Malboobi [a,*], Amir Mousavi [a]

[a] Department of Plant Biotechnology, National Institute of Genetic Engineering and Biotechnology, P.O. Box 14965/161, Tehran, Islamic Republic of Iran
[b] Department of Plant Breeding and Biotechnology, Faculty of Agriculture, Tarbiat Modares University, P. O. Box 14115-336, Tehran, Islamic Republic of Iran

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ABSTRACT

Purple acid phosphatase (PAP; EC 3.1.3.2) enzymes are metallophosphoesterases that hydrolysis phosphate ester bonds in a wide range of substrates. Twenty-nine PAP-encoding loci have been identified in the Arabidopsis genome, many of which have multiple transcript variants expressed in response to diverse environmental conditions. Having analyzed T-DNA insertion mutants, we have provided strong pieces of evidence that *AtPAP9* locus encodes at least two types of transcripts, designated as *AtPAP9-1* and *AtPAP9-2*. These transcript variants expressed distinctly during the course of growth in medium containing sufficient phosphate or none. Further histochemical analysis by the use of *AtPAP9-1* promoter fused to β-glucuronidase reporter gene indicated the expression of this gene is regulated in a tissue-specific manner. *AtPAP9-1* was highly expressed in stipule and vascular tissue, particularly in response to fungal infection. Subcellular localization of AtPAP9-1:green fluorescent fusion protein showed that it must be involved in plasma membrane and cell wall adhesion.

Purple acid phosphatase (PAP) enzymes catalyze the hydrolysis of a wide range of phosphoric acid mono- or di-esters and anhydrides at acidic or neutral pH (Cox et al., 2007). The characteristic pink or purple color of purified PAP proteins is related to charge transitions between a tyrosine residue and chromophoric ferric ion in the binuclear center (Plaxton and Tran, 2011). Multiple sequence alignments of eukaryotic and prokaryotic PAPs revealed seven invariant residues contained in five blocks of conserved amino acid sequences required for metal coordination, **D**XG/G**D**XX**Y**/G**N**H(D/E)/VXX**H**/G**H**X**H** (bold letters represent metal-ligating residues; Li et al., 2002).

Structurally, plant PAP proteins are categorized into high molecular weight (HMW) and low molecular weight (LMW) phosphatases. The former is thought to be functional in homodimeric form while the latter is typically monomeric carrying only metallophosphoesterase motifs (Klabunde et al., 1996). As contrasted to functional monomeric LMW ones, the biological importance of a fibronectin motif within the N-terminus of HMW PAPs is still unknown.

Our current knowledge suggests other functions for some plant PAPs as well. For example, AtPAP17 and AtPAP26 have both acid phosphatase and alkaline peroxidase activity. They could be involved in phosphate ion (Pi) scavenging and recycling as well as the metabolism of reactive oxygen species (del Pozo et al., 1999; Veljanovski et al., 2006). In comparison, AtPAP12 secreted by Pi-deficient Arabidopsis suspension cells and seedlings was highly active against several Pi-ester substrates over a broad range of pH range, making it ideally suited for scavenging Pi from the organic-Pi pools prevalent in many soils (Tran et al., 2010).

The expression of several PAP-encoding genes in response to available Pi has been documented by several researchers (for example, see Lohrasebi et al., 2007; Misson et al., 2005; Morcuende et al., 2007; Wu et al., 2003). Increased expressions of *PAP* genes have also been reported in response to other environmental conditions including wounding, nematodes, insects, high NaCl, oxidative stresses and senescence (del Pozo et al., 1999; Feng et al., 2003; Jakobek and Lindgren, 2002; Liao et al., 2003; Liu et al., 2005; Lohrasebi et al., 2007; Williamson and Colwell, 1991).

Recent studies have illustrated that PAP proteins are localized in various cellular components where they play different roles. For instance, AtPAP2 was localized in both plastids and mitochondria outer membrane (Sun et al., 2012). AtPAP12 and AtPAP10 were secreted out of the root cell (Tran et al., 2010; Wang et al., 2011) while AtPAP26 and AtPAP18 were dually targeted to vacuole and extracellular space (Tran et al., 2010; Zamani et al., 2012).

In addition, it is well known that alternative-first-exon transcriptions as well as alternative splicing processes allow

* Corresponding author. Tel./fax: +98 21 44580369.
E-mail addresses: katayounzamani@yahoo.com (K. Zamani), narcis@nigeb.ac.ir (T. Lohrasebi), ssabet2000@yahoo.com (M.S. Sabet), malboobi@nigeb.ac.ir (M.A. Malboobi), m-amir@nigeb.ac.ir (A. Mousavi).

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generation of large repertoire of proteins from a limited number of genes, enabling plants adaptation to changing environmental conditions (for a review see Reddy, 2007). Li et al. (2002) reported the existence of 29 loci within Arabidopsis genome encoding PAPs for which some preliminary expression data were presented. They also recorded two transcript variants for *AtPAP9* gene located at At2g03450 locus at GenBank (Ac. Nos. AF492661 and AY090895). However, they did not provide any experimental evidence for the existence of these two transcripts and the expression level of each one.

In the present work, we have shown the existence of two mRNA variants for *AtPAP9* gene in *Arabidopsis*, named *AtPAP9-1* and *AtPAP9-2*, by analyzing T-DNA knock-out lines. With this assumption, the expression patterns of the corresponding transcripts were compared in growing seedlings. As the variants differ for the N-terminal parts only, the importance of this sequence was investigated by further analysis of AtPAP9-1. Besides, the natures of *cis*-elements present in the *AtPAP9-1* promoter leading to its responsiveness to biotic and abiotic stimuli were analyzed at tissue level. Having illustrated the subcellular location of AtPAP9-1 protein, this is the first report indicating a potential role in plasma membrane and cell wall adhesion for N-terminal domain of a HMW PAP protein.

1. Results and discussion

1.1. AtPAP9 mutation analysis

Collections of T-DNA mutants of Arabidopsis were searched for disruptions in *AtPAP9* locus. Two lines were identified with T-DNA insertion in the first and the second exons, named SALK_129905 and SALK_020806, respectively. Progenies of SALK_129905 line were genotyped by multiplex PCR method using a pair of *AtPAP9* specific primers plus a primer derived from the T-DNA left boarder (Fig. 1A). Three homozygous plants were found for disruption in the first exon (Fig. 1B). However, no homozygous progeny for this line was found when over 130 progenies of self-pollinated heterozygote SALK_020806 parents were examined in the same way (Fig. 1C and D). Therefore, we proposed that SALK_020806 mutant possesses a recessive lethal trait. The segregation ratio close to 1:2 for wild type and the heterozygote progenies were in favor of this hypothesis. This was further supported by the examination of immature siliques of the self-pollinated heterozygous plants in which there were a numbers of empty slots carrying aborted ovules and aborted shrunken brown seeds (Fig. 1E).

The phenotypes of T-DNA insertion lines SALK_129905 versus wild type plants were compared on solid media containing sufficient or no Pi. Unlike SALK_020806 mutant, no detectable difference was noted in the growth rates or the appearance of shoots and roots of three-week old hetero/homozygote SALK_129905 mutants and wild type plants (data not shown). The above observations could only be explained by assuming the expression of an alternative transcript derived from the second exon in SALK_129905 helps plant survival. Its corresponding protein must have an important function that led to lethality if lacking.

1.2. Structural features of the AtPAP9 proteins

Having a molecular weight of 74 kDa, AtPAP9-1 is classified as a HMW PAP with a noncatalytic domain at the N-terminal and a catalytic domain at C-terminal. As shown in Fig. 2, detailed analysis of AtPAP9-1 sequence revealed several important features including a signal peptide sequence at the N-terminus with 20 amino acid residues, an RSGD motif (position 33 to 36), a DLXXL motif (position 50 to 54) as recognition site of αvβ6 integrin (Kraft et al., 1999), two DGE motifs (position 165 to 167 and 568 to 570) as recognition site for α2β1 integrin (Coulson et al., 1997), a fibronectin type 3 (FNIII) domain (position 141 to 239), a metallophosphatase domain (position 254 to 487) and a transmembrane region (position 604 to 626). The T-DNA fragments were inserted upstream of the FNIII domain and transmembrane region in SALK-129905 line and SALK_020806, respectively (Fig. 2).

1.3. Expression patterns of AtPAP9 transcript variants

Preliminarily, a comparative RT-PCR experiment was performed with cDNA molecules derived from wild type and the homozygous SALK_129905 seedlings using specific primer sets designed to amplify transcripts derived from each of the two exons (Fig. 3A). The amplification of two RT-PCR products with the expected sizes in wild type transcriptome and only one product corresponding to

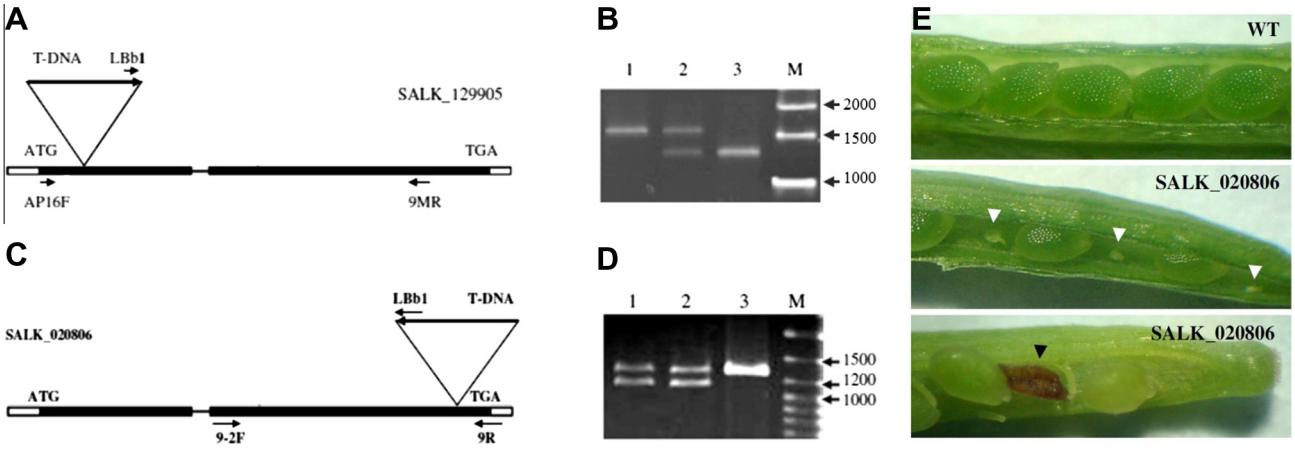

Fig. 1. Characterization of T-DNA insertion line SALK_129905 and SALK_020806. (A and C) The structure of the *AtPAP9* gene carrying T-DNA insert in the first and the second exons. Black boxes and line indicate exons and intron, respectively. The size of T-DNA is not drawn to scale. The locations of primers used for PCR genotyping (AP16F, 9-2F, 9MR, 9R and LBb1) are marked with arrows. (B) Illustration of homo/heterozygous T-DNA insertion loci by PCR on genomic DNA isolated from: (1) a wild-type plant; (2) a heterozygous mutant plant; and (3) a homozygous mutant plant. (D) Illustration of homo/heterozygous T-DNA insertion loci (SALK_020806) by PCR on genomic DNA isolated from: (1 and 2) heterozygous SALK_020806 plants; and (3) a wild type plant. M, 100-bp ladder DNA size marker. (E) Light microscopy of a wild-type immature siliques showing uniform seed development and heterozygous crossing SALK_020806 immature siliques containing aborted shrunken ovules (black arrowheads) and aborted brown shriveled seeds (white arrowheads).