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ORIGINAL ARTICLE

The *Burholderia pyrrocinia* Purple Acid Phosphatase Pap9 Mediates Phosphate Acquisition in Plants

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Abstract Increasing the expression level of purple acid phosphatases (PAPs), which hydrolyze organic phosphate to inorganic phosphate (Pi), is an important response to Pi starvation in plants. PAPs are widely distributed in eukaryotes like mammals and plants but distributed in limited microorganisms. A PAP-like protein named Pap9 from *Burkholderia pyrrocinia* CH-67 was isolated. The enzyme had optimal activity at atypical pH of 8.5 and temperature of 85°C. When *pap9* was overexpressed in *Arabidopsis thaliana*, the APase activity in the transgenic plants was about 15% to 40% higher under acidic conditions and had an approximately four-fold increase under alkaline conditions compared with wild type. Overexpression of *pap9* in plants enhanced APase activity under Pi-deficient conditions more than under Pi-sufficient conditions. In-gel assays revealed that Pap9 exists as a monomer in transgenic plants. Root surface-associated APase activity in transgenic plants increased dramatically during acidic and Pi-deficient conditions. These results indicated that bacterial Pap9 in transgenic plants was secreted onto the root surface and released into the rhizosphere. The transgenic plants have significantly enhanced biomass that have the potential for use in biotechnological applications.

Keywords: APase activity, Pi-deficient, Pi-sufficient, Purple acid phosphatase, Transgenic plants

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Introduction

Phosphorus is an essential nutrient element for plant growth, but it could be utilized only as the form of soluble inorganic phosphate (Plaxton and Tran 2011). Coping with phosphate nutritional stress, plants have evolved a variety of adaptive response pathways to improve Pi acquisition and utilization, such as root morphological changes, the induction of Pi transporters, the secretion of organic acids, phosphatases and ribonucleases (Tian et al. 2012).

Acid phosphatases catalyze the hydrolysis of inorganic phosphate from phosphoric compounds at a typically optimal acidic pH (Tran et al. 2010; Hurley et al. 2010; Tian et al. 2012). PAPs are a distinct group of APases that display a pink or purple color in solution (Tran et al. 2010). All members of this group contain seven metal-ligating sites on five conserved motifs (G**D**XX/G**D**XX**Y**/G**N**H(D/E)/XXX**H**/G**H**X**H**) (Olczak et al. 2003). PAPs are widely distributed in mammals and plants, but only a limited number exist in microorganisms (Schenk et al. 2000). The first purified microbial PAP was derived from a fungus, *Aspergillus ficuum*, and its sequence was closely related to plant HMW (high molecular weight, ~55 kDa) PAPs.

In silico analysis revealed that three bacterial genera, *Burkholderia*, *Mycobacteria* and *Frankia*, contain PAP sequences most like plant HMW PAPs (Yeung et al. 2009). The genus *Burkholderia* belongs to *Betaproteobacteria* and comprises more than 80 species with incredible functional diversity and distribution, from a wide range of environmental niches (Depoorter et al. 2016). We previously isolated and named *pap9*, a gene encoding a PAP-like protein located in contig 009 of the *B. pyrrocinia* CH-67 genome (Song et al. 2012). The strain was isolated from forest soil as a biocontrol

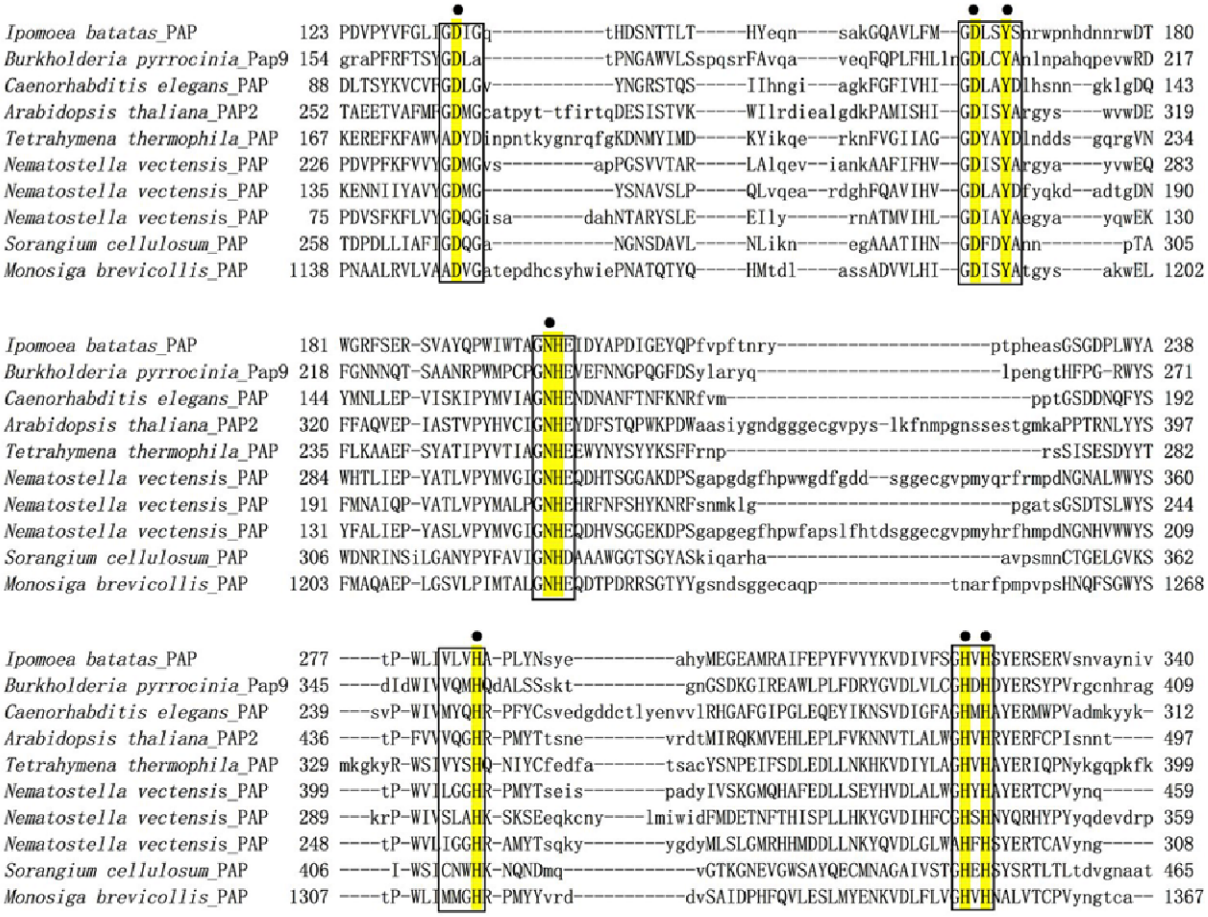

Fig. 1. The amino acid sequence alignment of Pap9 with corresponding PAP sequences from nonbacterial sources. The five conserved motifs are boxed in black rectangles. Yellow highlighting indicates active sites, and • indicates metal-binding sites.

agent (Lee et al. 2011). We investigated the heterologous expression and enzymatic characterization of *pap9* and introduced this bacterial *PAP*-like gene into Arabidopsis. The purpose of this study was to generate transgenic plants that have improved tolerance of Pi-deficient conditions for potential biotechnological applications.

Results

Bioinformatic Analysis of the Candidate *acp* Gene

In the genome of *B. pyrrocinia* CH-67, three genes encoding candidate acid phosphatases (*acp* genes) were found and cloned into *E. coli* DH5α to test APase activity by BCIP staining (Fig. S1), the *acp9*-transformed bacterial cells showed the strongest color. The full-length ORF of *acp9* was composed of 1,689 bp, encoding a polypeptide of 563 amino acids (WP_017329228.1). The molecular mass was approximately 60.9 kDa, with an isoelectric point of 5.87. Acp9 contained a purple acid phosphatase-like domain and a twin-arginine signal peptide consisting of 47 amino acids at its N-terminal, analyzed by Interproscan server. The five conserved motifs (G**D**LA/G**D**LC**Y**/G**N**HE/VQM**H**/G**HDH**) and the seven metal-ligating sites (D165, D199, Y203, N237, H354, H391, H393) of plant and animal PAPs were all identified in Acp9 by the alignment of the amino acid sequences (Fig. 1). Hence, this gene was named *pap9*.

Phylogenetic analysis of this protein together with other prokaryotic and eukaryotic PAP-like sequences was shown in Fig. S2, PAP-like sequences from the species in *Burkholderia*, *Paraburkholderia*, and *Caballerionia* which were highly similar to Pap9 were clustered together and distinct from the other species. The *Burkholderia* cluster had two small branches which were classified into the *B. cepacia* complex and *pseudomallei* group and corresponded with the 16S rRNA-based phylogenic tree of this genus (Depoorter et al. 2016). For *Paraburkholderia* and *Caballeronia*, the species were also clustered into distinctly separate groups corresponding to genera.

Biochemical Properties of Pap9

Acid phosphatase preferred to use pNPP substrate when

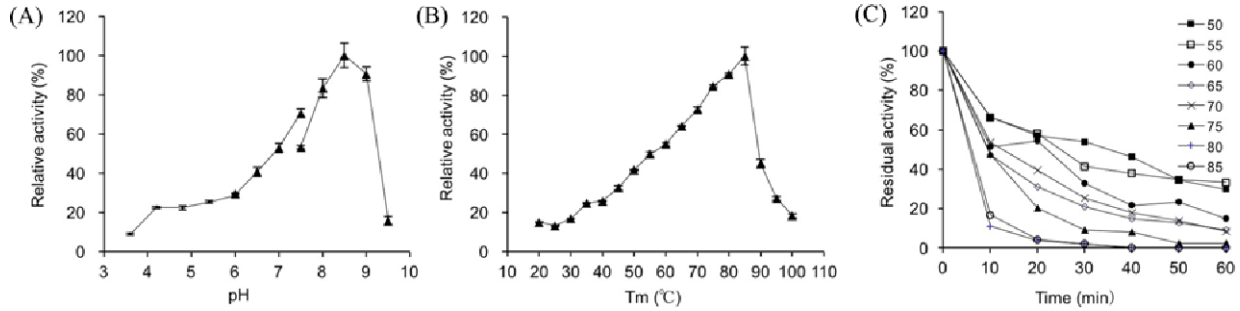

Fig. 2. Assays of Pap9 activity. (A) Enzyme activity at different pH. The enzyme activity was measured in the buffers containing 1 mM pNPP: sodium acetate buffer (pH 3.0 to 6.0), Tris-maleate buffer (pH 6.0 to 7.5), Tris-Cl buffer (pH 7.5 to 9.5). (B) The enzyme activity was measured from 20°C to 100°C at optimal pH 8.5. (C) Thermostability, the residual activity was measured by the standard APase assay after different heat treatments (50°C to 85°C) for 10 to 60 min, respectively.

increased in reaction temperature (Tham et al. 2010). Activity of acid phosphatase which hydrolyses pNPP to p-nitrophenol, depended on the temperature of the reaction (Peterson et al. 2004). To investigate optimal temperature, pH, and enzyme stability of PAP9, we assayed the enzyme activity using purified recombinant PAP9 protein. In Fig. S3, SDS-PAGE analysis showed that the molecular mass of Pap9 fusion protein was about 85 kDa which is equal to the predicted molecular sizes of Pap9 (60.7 kDa) and GST (26.1 kDa). To test the optimal pH for PAP9 enzyme activity, PAP9 protein was made at three different buffers at pH levels: sodium acetate buffer (pH 3.0 to 6.0); Tris-maleate buffer (pH 6.0 to 7.5); Tris-Cl buffer (pH 7.5 to 9.5) (Fig. 2A). The optimum pH for Pap9 activity was pH 8.5, while typical PAPs favor acidic conditions (Fig. 2A). To test the optimal temperature for PAP9 enzyme activity, pNPP was used as substrate (Fig. 2B). In Fig. 2B, the activity of Pap9 increased from 20°C to 85°C, with the highest activity at about 85°C. However, it fell sharply at temperatures over 85°C (Fig. 2B). To test thermostability of PAP9 enzyme, PAP9 protein was treated at 50 to 85°C from indicated time points before measuring enzyme activity (Fig. 2C). The results showed that the effects of temperature on the stability of Pap9. Pap9 lost half its activity after heat treatment at 50°C for 60 min, but activity was still detected after 60 min at 75°C (Fig. 2C). Although Pap9 displayed its highest activity at a relatively

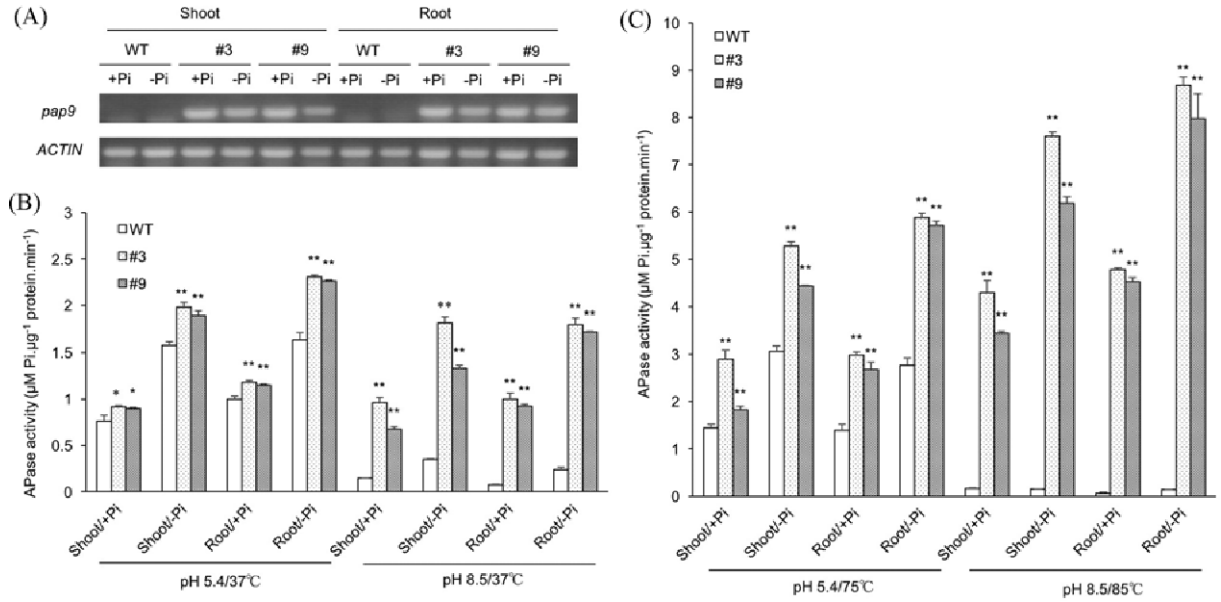

Fig. 3. Expression analysis of *pap9* in WT and transgenic plants under +Pi and –Pi conditions. (A) RT-PCR analysis, 5-d-old seedlings were transferred onto +Pi and –Pi MS solid medium for 7 days. Shoot and root tissues were ground separately with liquid nitrogen to extract total RNA. *ACTIN* served as internal control. (B) and (C), Phosphatase activity assay of transgenic plants. Shoot and root tissues were ground separately with liquid nitrogen to extract proteins for analysis. The hydrolysis of pNPP at four different reaction conditions: pH 5.4/37°C, pH 8.5/37°C (B) and pH 5.4/75°C, pH 8.5/85°C (C). WT, wild type; #3 and #9, transgenic lines. Values represent means with standard deviation of three replicates. * indicates that the value is significantly different from wild type (*; 0.01 < p-value ≤ 0.05, **; p-value < 0.01, Student's t-test).

high temperature (85°C), it was not very stable at this temperature (Fig. 2B, C). These data suggested that optimal temperature and pH were able to dramatically increase the enzyme activity of PAP9, although enzyme themostability of PAP9 lost during the duration at high temperature.

Characterization of *pap9* Transgenic Arabidopsis Plants

As Pap9 was thought to be an atypical HMW PAP compared to most plant PAPs, the *pap9* gene was introduced into plants to investigate whether it might be beneficial to them. Firstly, RT-PCR analysis (Fig. 3A) indicated that the bacterial *pap9* gene was introduced into plants successfully and could be expressed in transgenic plants under the the control of the *CaMV* 35S promoter.

Then, the APase activity of shoot and root protein extracts of wild type and transgenic plants was detected under four different reaction conditions, pH 5.4/37°C, pH 8.5/37°C, pH 5.4/75°C and pH 8.5/85°C, as Pap9 appeared to be a heat-stable alkaline phosphatase. The total APase activity in shoots and roots of WT and *pap9* transgenic plants was about two-fold higher under Pi-deficient conditions (Fig. 3B, C). These results indicated that Pi starvation induced enzyme activity of the plants APases and Pap9 simultaneously. APase activity was about 15% to 40% higher in transgenic plants than that in the WT at pH 5.4/37°C. APase activity was reduced by more than 80% in the WT, while its activity in transgenic plants was reduced by less than 30%, under alkaline conditions (Fig. 3B). These results revealed that most plant APases are acid phosphatases and that Pap9 contributed mainly to the activity of APase in transgenic plants. As shown in Fig. 3C, although the APase activity in WT increased at this high temperature, the APase activities of transgenic lines were about twice those of the WT under both Pi conditions. The APase activity of the WT was barely detectable at pH 8.5/85°C, however, the transgenic plants showed the highest activity under this condition, compared to the other three reaction conditions.

The in-gel assay profile of APase activity in root extracts was tested at pH 5.4 and pH 8.5 simultaneously (Fig. 4). Four APase isoforms, designated A1, A2, A3 and A4, were observed. The color of the bands was dramatically increased under Pi-deficient conditions, which was consistent with the results from the enzymatic reaction (Fig. 3B). A1 and A2 bands were observed in both WT and *pap9* transgenic plants, and they displayed a more intense color at pH 5.4. These results suggested that these two bands represented the intrinsic APases of Arabidopsis, induced by phosphate starvation. In *pap9* transgenic plants, two new A3 and A4 isoforms with molecular masses around 70 kDa, which exhibited enhanced color at pH 8.5, were detected, suggesting that these two bands represented the transgenic Pap9 proteins. However, the precise molecular mass of the two bands could not be determined in the in-gel assays because the protein extracts were electrophoresed using native-PAGE gels. The predicted molecular mass of the Pap9 protein is about 60 kDa, indicating that Pap9 exists as a monomer in *pap9* transgenic plants. Pap9 showed two isoforms, possibly induced by some posttranscriptional modification event, such as glycosylation.

Secretion of Pap9 in roots of transgenic plants

To investigate whether Pap9 is secreted from the root, an in vivo root BCIP staining activity assay was performed. As shown in Fig. 5A, even at pH 5.4, transgenic plants exhibited higher APase activities than the WT, and the activities were substantially enhanced under –Pi conditions. On the other hand, because Pap9 has low activity under relatively low

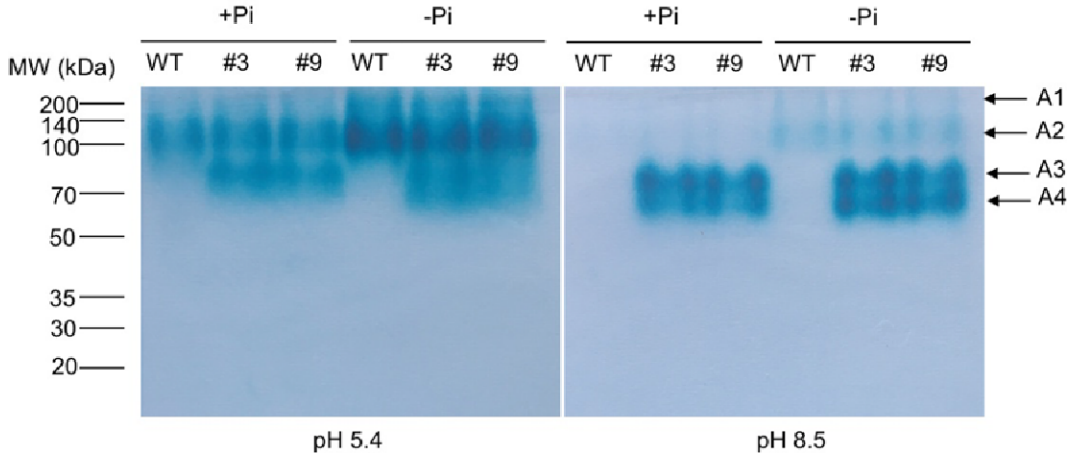

Fig. 4. In-gel assay profile of root extracts. Seven-day-old seedlings were cultured in +Pi and –Pi liquid medium for more seven days. Protein extracts of root were separated by native-PAGE gels and then stained by BCIP with pH 5.4 and pH 8.5 buffer, respectively. Four APase isoform bands were observed and designated A1, A2, A3, and A4. WT, wild type; #3 and #9, transgenic lines.

temperatures (< 50°C) (Fig. 2B), both WT and *pap9* transgenic plants under +Pi conditions exhibited white roots at pH 8.5 when BCIP was applied at a typical temperature for plant culture (23°C). However, during Pi starvation, only the *pap9* transgenic plants showed a weaker but clearer color than the WT. These results suggested that most root-associated APases in Arabidopsis are acid phosphatases, and further proved that the activity of bacterial *pap9* in transgenic plants could be induced by phosphate starvation.

To confirm whether Pap9 in transgenic plants could be secreted as a root-associated APase or released into the culture medium, the APase activities between the WT and *pap9* transgenic plants under +Pi and –Pi conditions were detected (Fig. 5B). The total APase activity of the *pap9* transgenic plants in the culture medium was much higher (approximately 0.4- to 2-fold) than that of the WT under both Pi conditions (Fig. 5B). Moreover, in both +Pi and –Pi conditions, the root-associated APase activity of *pap9* transgenic plants was weakly higher (approximately 0.15- to 0.45-fold) than that of WT plants (Fig. 5B). These datas indicated that bacterial PAP9 mediated the total APase activity in the culture medium as well as the root-associated APase activity in root surface, regardless of +Pi and –Pi. In addition, as was the case with the *35S:AtPAP12* and *35S:AtPAP26* transgenic plants, bacterial PAP9 in transgenic plants was mostly associated and secreted onto the root surface, enhancing root-associated APase activity (Wang et al. 2014). The root-associated APase activities in WT and PAP9 transgenic plants, regardless of +Pi and –Pi, were significantly higher than the total APase activity in the culture mediums, indicating that most plant APases were associated and secreted with the root surface compared to release into the rhizosphere (Fig. 5B, C). Since Pap9 is an alkaline phosphatase and the culture medium was acidic, acetate buffer (pH 5.4) and Tris-Cl

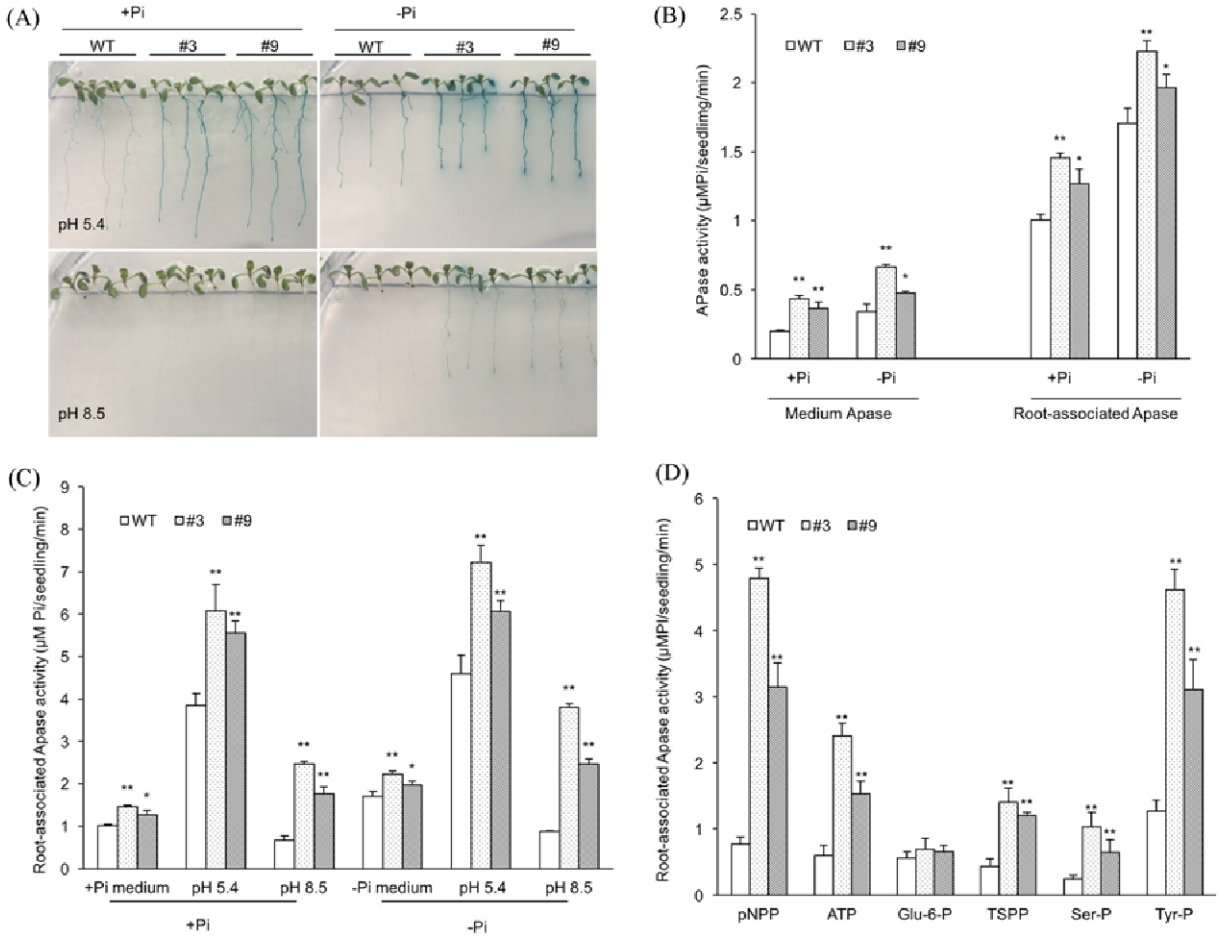

Fig. 5. Root-associated APase activity in WT and *pap9* transgenic plants under +Pi and –Pi conditions. (A) APase activity detection by BCIP staining of the roots of WT and *pap9* transgenic plants grown on +Pi and –Pi medium. (B) Total APase activity of culture medium and root surface of WT and *pap9* transgenic plant seedlings. (C) Root-associated APase activity was detected in medium and sodium acetate buffer (pH 5.4) and Tris-Cl buffer (pH 8.5) respectively. (D) The activities of root-associated APases of WT and *pap9* transgenic plants towards several substrates under pH 8.5, glucose-6-phosphate (Glu-6-P), pyrophosphate tetrabasic (TSPP), O-phosphoserine (Ser-P), O-phosphotyrosine (Tyr-P). WT, wild type; #3 and #9, transgenic lines. Values represent means with standard deviation of three replicates. * indicates that the value is significantly different from wild type (*; 0.01 < p-value≤0.05, **; p-value < 0.01, Student's t-test).

Table 1. Relative activity of Pap9 with different substrates

Substrate	Relative activity (%)
pNPP	100
ATP	11.7±2.0
Glucose-6-phosphate	3.6±0.4
Phyrophosphate	3.7±1.0
Sodium phytate	0.5±0.1
O-phosphoserine	20.8±0.7
O-phosphotyrosine	54.3±5.7

The final concentration of substrates was 1 mmol.L-1. The relative activity was shown as the percentage of the activity against pNPP at 37°C (100%, 106.23±3.62 μkat/g). Values represent means with standard deviation of three replicates

buffer (pH 8.5) were also used as reaction buffers for the root-associated APase activity assays of seedlings (Fig. 5C). The APase activity detected in sodium acetate buffer (pH 5.4) was about three to four times higher than that using culture medium as buffer (pH 5.7), however, the trends in activity between WT and *pap9* transgenic plants were similar. The root-associated APase activity in *pap9* transgenic plants under alkaline conditions was about three to four times greater than that in WT plants. These results indicated that most APases in plants are acidic. In addition, the combined results suggested that *pap9* transgenic plants have enhanced Apases activity under both acidic and alkaline conditions that they could increase phosphate utilization possibly.

The total root-associated APase activity was measured using organophosphate compounds (Table 1) as substrates under alkaline conditions (Fig. 5D). Because most APases in WT plants can display very low activity under alkaline conditions, the trend in root-associated APase activity in *pap9* transgenic plants was consistent with the activity of pure Pap9 protein (Table 1). This suggested that Pap9 contributes mainly to the total root-associated APase activity under alkaline conditions in transgenic plants.

The Growth Appearance and Phosphorus Utilization of Transgenic Plants

Overexpression of *pap9* in Arabidopsis could improve the APase activity of transgenic plants under Pi limitation, so we wonder the ability of phosphorus utilization in transgenic plants. Five-day-old seedlings were cultured on the mediums containing 50 μM *O*-phosphotyrosine as the sole P source for two weeks. As the result, both WT and transgenic plants

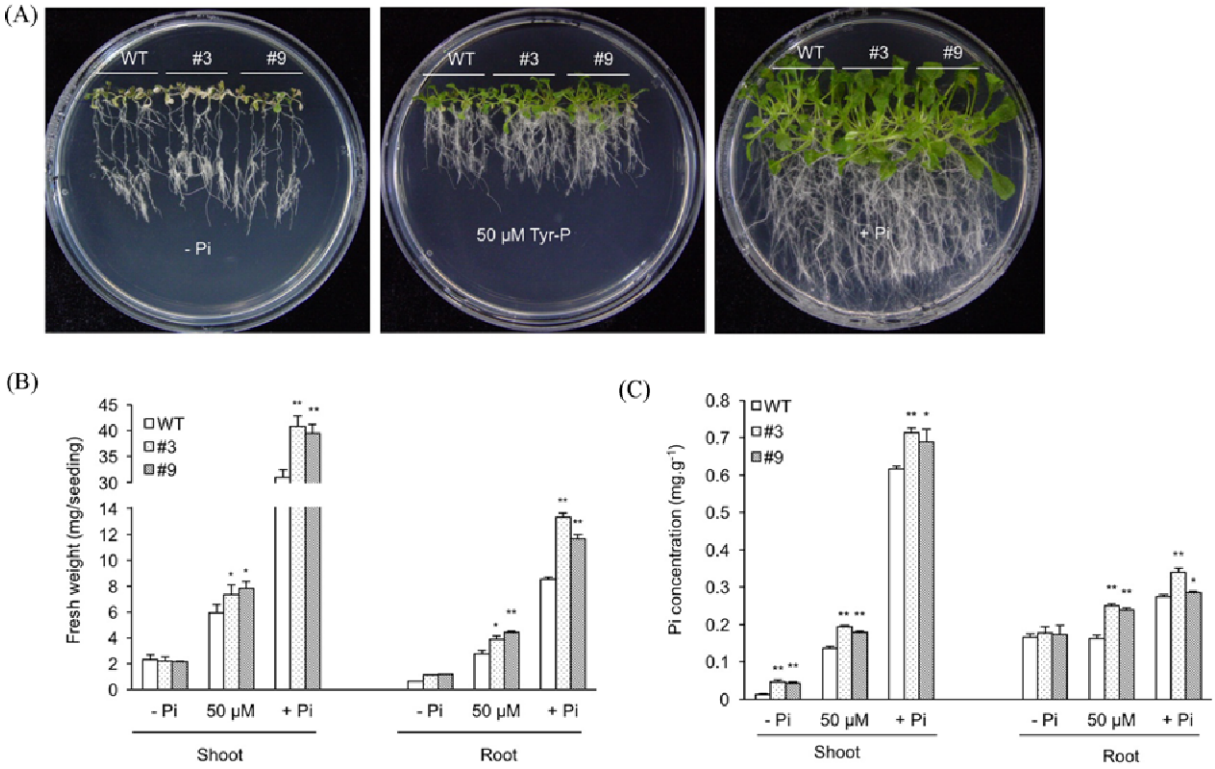

Fig. 6. Growth characteristics of WT and *pap9* transgenic plants under different phosphate conditions. (A) The growth appearance. (B) Shoot and root fresh weight. (C) The available phosphate concentration in shoot and root. Five-day seedlings transferred onto –Pi, +Pi medium and –Pi medium supplemented with 50 μM *O*-phosphotyrosine and cultured for more two weeks. WT, wild type; #3 and #9, transgenic lines. Values represent means with standard deviation of three replicates. * indicates that the value is significantly different from wild type (*; 0.01 < p-value ≤ 0.05, **; p-value<0.01, Student's t-test).

could utilize *O*-phosphotyrosine because they grew better along with the substrate concentration increase (Fig. 6A). However, the shoot and root biomasses of transgenic plants were both significantly higher than WT when supplying with 50 μM *O*-phosphotyrosine as P source (Fig. 6B). The Pi concentrations in shoots of transgenic plants were significantly higher than those of WT (Fig. 6C), no matter whether phosphorus was supplied. The results indicated that prokaryotic Pap9 could also improve the utilization of extracellular phosphorus.

Discussion

Distribution of PAP-like Sequences in Prokaryotic Genomes

PAP-like sequences only distribute in a few prokaryotes. Moreover, the low similarity between the protein sequences indicated that prokaryotic PAPs may have high sequence diversity, indicating potentially divergent functions. However, it is worth mentioning that the identified PAP sequences widely exist in *Burkholderia, Paraburkholderia*, and *Caballerionia*, all of which were identified previously as *Burkholderia* and now belong to *Burkholderia* sensu lato (Estrada-de et al. 2018). *Burkholderia* sensu lato was split to distinguish pathogenic and plant beneficial and environmental species (Estrada-de et al. 2018). Even though the division of *Burkholderia* sensu lato was controversial and will continue to experience changes as more sequenced genomes are analyzed, the clades of Pap9-like sequences in this large group correspond with the 16S rRNA-based phylogenetic tree. This suggests that the evolution of *Burkholderia* Paps is related to the diversity of *Burkholderia*, caused by the host environment, and that Paps could be used as markers for taxonomy and identification.

Enzymatic Characterization of Pap9

Although the sequence of Pap9 was similar to those of plant PAPs, the APase activity of the prokaryotic Pap9 is optimal under alkaline conditions (Fig. 2A). Among prokaryotic PAPs, *Burkholderia cenocepacia* PAP (BcPAP) also has optimal activity in the alkaline pH range (Yeung et al. 2009). As most PAP-like sequences of *Burkholderia* sensu lato have high identity, we suggested that they may represent a novel sub-class of PAPs. Despite the differences in optimum pHs, BcPAP mediated Pi acquisition to the same extent as plant PAPs (Yeung et al. 2009). Since *B. pyrrocinia* CH-67 is associated with plant growth and protection, Pap9 might have evolved to benefit the plant.

Physiological Role of Pap9 in Plants

PAPs in plants play roles in various biological functions including developmental and stress responses, such as flower development, cell wall biosynthesis, oxidative damage, salt stress, and phosphate starvation (Liao et al. 2003; Zhu et al. 2005; Zhang et al. 2008; Kaida et al. 2009). PAPs were found to contribute to the regulatory function of several sophisticated mechanisms to enhance Pi acquisition in plants, regardless of their origin (Liang et al. 2010), such as common bean (*Phaseolus vulgaris*) PvPAP3 (Liang et al. 2010), Arabidopsis AtPAP26 (Hurley et al. 2010) and AtPAP10 (Wang et al. 2011), soybean (*Glycine max*) GmPAP4 (Kong et al. 2014) and GmPAP21 (Li et al. 2017), rice (*Oryza sativa*) OsPAP10c (Lu et al. 2016) and OsPAP21b (Mehra et al. 2017), all PAPs of which were increased under Pi deficient-conditions to improve Pi availability.

Even though the bacterial Pap9 seems to be an atypical PAP which has an alkaline optimal pH, the APase activity in *pap9*-overexpressing plants was increased under both acidic and alkaline pH conditions, and that the phosphate utilization in transgenic plants were also improved under phosphate-deficient conditions. These suggest that *pap9* transgenic plants have the potential for use in biotechnological applications, such as in transgenic plants grown in saline, alkaline soils, especially.

Recent studies reported that bacterial genes in plants were able to function in various physiological processes in growth, development, and stress adaptation (Buée et al. 2009; Peiffer et al. 2013). Our results suggest that in heterologous systems, Pap9 may play an important physiological role in the Pi starvation response by maintaining APase activity. Physiological characterization of other prokaryotic PAPs in Pi acquisition or recycling during Pi starvation is also of interest. Most importantly, we need to investigate if the *pap9* gene will benefit field-grown plants under different environments, e.g. under low-phosphate or other abiotic stress conditions.

Materials and Methods

Bacteria and Plant Materials and Growth Conditions

B. pyrrocinia CH-67 was previously isolated from forest soil. *Escherichia coli* DH5a and BL21 (DE3) were used as cloning and expression hosts, *Agrobacterium tumefaciens* GV2260 was used in Arabidopsis transformation. The ecotype of *Arabidopsis thaliana* was Columbia-0 background. Seeds were surface sterilized and sown on MS solid medium and kept horizontally in a growth room at 23°C after 2 days-stratified at 4°C. The Pi-sufficient (+Pi) or -deficient (-Pi) medium was Murashige and Skoog (MS), including 2% sucrose and pH 5.7 with 1.25 mM PO_4^{3-} (+Pi) or 12.5 μM PO_4^{3-} (-Pi).

Bioinformatics Analysis

Amino acid sequence was used to enquiry in NCBI non-redundant protein database by PSI-BLAST (Position-Specific Iterated BLAST) search. Amino acid sequences alignment was done by Clustal W program. The phylogenetic tree was constructed by the neighbor-joining (NJ) method of MEGA7 program. Prediction of the molecular

weight of proteins could be realized by ExPASy server.

Gene Cloning, Protein Expression and Purification

The primers were designed for pap9 cloning: *pap9*-F (*Bam*HI), 5'-*GGATCC*ATGTCGAACCCGGACAAC-3'; *pap9*-R (*Hin*dIII), 5'-*AAGCTT*TTACCGTTCGCGGCGCTT-3'. The target fragment was cloned into pGEM-T easy vector (Promega, Madison, USA) and then sub-cloned into pET-42a(+) vector which contains a GST (glutathione S-transferase) tag, and transformed into *E. coli* strain BL21(DE3). The protein expression condition: 1 mM IPTG (isopropyl β-D-1-thiogalactopyranoside) induction for 4 hours at 28°C. GST-fusion protein purification was according to the manual supported by GE Healthcare with 1 ml GSTrap FF column. Finally, the GST-fusion proteins were eluted by 50 mM Tris-HCl (pH 8.0) containing 10 mM reduced glutathione. In order to achieve pure PAP protein, the mixture of 80 µl factor Xa protease (80 U) and 920 µl buffer (50 mM Tris-Cl, 150 mM NaCl, 2 mM $CaCl_2$, pH 7.5) was loaded into the GSTrap column to digest and remove GST tag at room temperature overnight before elution during purification. The protein concentration was determined by Bradford method incubating with Protein Assay dye reagent concentrate (Bio-Rad, Berkeley, USA). SDS-PAGE and Western Blotting were used to further determine the quality of purified Pap9. The primary anti-GST-tag monoclonal mouse antibody and the secondary HRP conjugated goat-anti-mouse IgG antibody (Novagen, Madison, WI, USA) were used for Western Blotting.

Phosphatases Activity Assay

Enzyme activity assay was carried out in 100 mM sodium acetate buffer (pH 5.4) containing 1 µg of Pap9 protein and 1 mM pNPP (*p*-nitrophenol phosphate) substrate. After 30 min water bath at 37°C, the reaction was terminated by adding 0.1 M NaOH, then the absorbance at 410 nm was measured. The unit of phosphatase activity (kat) was defined as the conversion of one mole of substrate per second.

Biochemical Characterization of Pap9 Protein

For the optimal pH of Pap9, enzyme activity was determined under three kinds of buffers: sodium acetate buffer (pH 3.0 to 6.0), Tris-maleate buffer (pH 6.0 to 7.5), Tris-Cl buffer (pH 7.5 to 9.5). For the optimal temperature, enzyme activity was tested from 20°C to 100°C under pH 8.5. For the thermal stability of Pap9, the residue enzyme activity was carried out after heat treatment: samples were heat under different temperature (50°C to 85°C) for a period of times (10 to 60 min) respectively. After water bath treatment, samples were kept onto ice immediately to stop the inactivation process, and the remaining activity was determined by pNPP method as described above. In order to analyze the substrate specificity, glucose-6-phosphate (Glu-6-P), sodium phytate, β-glycerophosphate, pNPP, sodium pyrophosphate tetrabasic (TSPP), O-phosphoserine (Ser-P), O-phosphotyrosine (Tyr-P), and ATP were selected as substrates, and the enzyme activity was carried out by determining the content of released Pi based on the according to the method of Fiske and Subbarow (Freitas-Mesquita et al. 2014). Briefly, ammonium heptamolybdate was dissolved by 20% sulfuric acid to final concentrate 5%, this solution and 10% ferrous sulfate were used as assay buffers to determine Pi content after enzymatic action at 650 nm.

Construction and Confirmation of Transgenic Plant

The Gateway cloning method was applied to the construction of recombinant vectors. The whole nucleotide sequences with stop codon which were added with four bases "CACC" at 5' terminal by forward primers were introduced into the gateway vector pENTR. By LR reaction, the *pap9* were cloned into a destination vector pH7WG2D.1 which contains hygromycin gene. The constructed vector was transformed into *A. tumefaciens* GV2260 by electroporation. Flower vacuum infiltration procedure was used in the Arabidopsis transformation. Transgenic T1 plants were screened on 1/2 MS medium containing 20 mg L^{-1} hygromycin and 100 mg L^{-1} carbenicillin and and then transferred into soil. Finally, homozygous plants were selected for the further assay.

RT-PCR (Reverse transcription PCR)

RNA extraction was according to the instructions of a plant RNA purification reagent (Invitrogen, Carlsbad, CA, USA). The one-step RT-PCR was realized by SuPrimeScript RT-PCR kit (GeNet Bio, Daejeon, Korea). The expression of housekeeping gene *ACTIN* was as a control. The following primers were used for specific PCR: *pap9*-RT-F 5'-CGTGGCTTCCTGAAA- CTCGC-3' and *pap9*-RT-R 5'-GCTCGCGGTGAACGGTTG-3'; *AtACTIN*-F 5'-AGTGTGTC-TTGTCTTATCTGGTTCG-3' and *AtACTIN*-R 5'-AGCTGCATT-GTCACCCGATACT -3'.

Extraction and Quantitative APase Activity Assay of Plant Protein

The shoot and root samples were ground into powder with liquid nitrogen through TissueLyser, then suspended with protein extraction buffer (pH 5.4, 100 mM potassium acetate, 20 mM $CaCl_2$, 2 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride and 20% glycerol). Samples were gently located on ice for 30 min and then centrifuged at 14,000 g at 4°C for 20 min. The supernatant was transferred to a new tube and stored at -80°C. pNPP method described above was used to measure the total APase activities of shoot and root protein extracts.

In-gel Assay

Ten microgram plant protein samples were separated on 10% native-PAGE gel in the ice-water mixture. After electrophoresis, the gels were washed in double distilled water and then equilibrated with sodium acetate buffer (pH 5.4) and Tris-Cl buffer (pH 8.5) respectively. The gels were stained with 0.05% BCIP (5-bromo-4-chloro-3-indolyl-phosphate) dissolved in buffer (pH 5.4) and Tris-Cl buffer (pH 8.5) at 37°C overnight, respectively.

Root-associated APase Activity Assay

In vivo APase activity of root was detected by BCIP staining, the roots of seedlings were overlaid with the sodium acetate buffer (pH 5.4) and Tris-Cl buffer (pH 8.5) containing 0.6% agar and 0.01% BCIP overnight, respectively.

Wang et al. (2011) has described the method to determine root-associated APase activity in detail. Here, the assay was performed with some modifications. Seven-day-old seedlings in a consistent state were individually transferred to 2 ml tubes with +Pi and –Pi mediums for three days treatment. Seedlings were gently washed in sterilized deionized water and transferred to another tubes containing fresh +Pi and –Pi liquid mediums correspondingly. +Pi and –Pi treated seedling were also transferred to the tubes with sodium acetate buffer (pH 5.4) and Tris-Cl buffer (pH 8.5) respectively. Then 1 mM pNPP was added into all tubes to measure the APase activity as above. The APase activity against different substrates (listed in Table 1) was determined by adding 1 mM substrates into the tubes containing –Pi treated plants in Tris-Cl buffer (pH 8.5), respectively. The Pi released was measured by molybdenum blue method as above.

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Author's Contributions

XZ, SWL and DHK desigend the experiments; XZ performed most of the experiments; SYL performed part of the experiments; XZ and DHK wrote the manuscript; WTY and SWL discussed and commented on the results; DWB, SWL and ML discussed and commented on the manuscript. DHK provided fund for research work as corresponding author.

Supporting Information

Fig. S1. Simple confirmation of phosphatase activity by BCIP hydrolysis.
Fig. S2. Phylogenetic analysis of Pap9 and PAP-like phosphatases from various species.
Fig. S3. SDS-PAGE analysis of GST-Pap9 fusion protein and western blot confirmation.

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