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Full Length Research Paper

Molecular characterization and functional analysis of *OsPHY2*, a phytase gene classified in histidine acid phosphatase type in rice (*Oryza sativa* L.)

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Phytic acid (PA) and its derivatives contain the major portion of phosphorus in the cereal seed and chelates divalent cations. During germination, both minerals and phosphate are released upon phytase-catalyzed degradation of PA. In this study, a phytase gene classified into histidine acid phosphatase type in rice referred to OsPHY2 was characterized. *OsPHY2* is 2060 bp in length, encoding a polypeptide of 519 amino acids. OsPHY2 contains HP_HAP_like domain, a 24-aa signal peptide at the N-terminal and was targeted to the cytoplasm membrane after ER sorted. Phylogenetic analysis suggests that *OsPHY2* shares high similarity to the phytase genes in wheat and a subset of genes without characterized functions in other plant species. During the seed germination, the transcripts of OsPHY2 were much more detected in the germinated seeds than in the primary roots and the leaves. Prokaryotic expressed OsPHY2 has a strong function on degradation of phytate, with optimal temperature of 47°C and optimal pH of 3.5. Under the guidance of patatin signal peptide (PSP) of potato, the OsPHY2 expressed in tobacco could be secreted by the rhizosphere and hydrolyze the phytate into Pi, leading to a significantly more inorganic phosphate (Pi) accumulated and growth improved in plants under the condition using phytate as the sole phosphorus source. Therefore, as one HAP type of the phytase genes, *OsPHY2* plays a critical role on the degradation of the phytins during the seed germination and has a potential application on the generation of elite crop germplasms with high use of efficiency of phytate and a large quantity of organic phosphate compounds in the arable land.

Key words: Rice (*Oryza sativa* L.), phytase gene, expression, biochemical property, transgene analysis.

INTRODUCTION

As one of the indispensible macro-inorganic nutrients, inorganic phosphate (Pi) with adequate levels is critical to the growth and development of all organisms. Pi exerts a range of functions such as the components of the macromolecules and to be involved in the energy generation, and metabolic regulation. The demand for Pi increases dramatically during periods of rapid cell growth and division, such as seed germination.

In the cereal seeds, the phosphorus is mostly stored in forms of organic compounds, such as phytic acid (*myo*-inositol hexa*kis*phosphate) and its derivatives during seed development (Lott et al., 1995; Raboy, 1997). Owing to biochemical metabolism during the seed filling stage, the Pi in the seeds transferred from the growth medium and other tissues is transformed into the organic compounds, resulting in maximal levels of phytic acid and its derivatives at seed maturity (Raboy and Dickinson, 1987). During the seed germination, the phytin reserves in seeds are hydrolyzed to supply the nutrients for the rapidly growing seedling. It is noted that the dephosphorylation of phytin to a series of *myo*-inositol esters and inorganic phosphate is catalyzed by phytases that belong to a type of phosphatases (Loewus and Murthy, 2000). Thus far, totally four distinct classes of phytase have been characterized in the organisms including histidine acid phosphatases (HAPS), B-propeller

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Abbreviations: PA, Phytic acid; **HAPS,** histidine acid phosphatases.

phytases, purple acid phosphatases (Mullaney and Ullah, 2003), and protein tyrosine phosphatase-like phytases (PTP-like phytases) (Puhl et al., 2007). Of which, most of the known phytases belong to a class of HAPs. Thus far, most of the phytases have been isolated from filamentous fungi, bacteria and yeast (Kim et al., 2006; Chen et al., 2006; Golovan et al., 2006; Lim et al., 2007; Nakashima et al., 2007; Puhl et al., 2009).

In plants, phytases have been purified from the seeds of various monocot and dicot species (Wodzinski and Ullah, 1996). During seed germination, a pronounced increase in phytase activity was induced in soybean, accompanying a concomitant decrease in phytic acid, with maximal phytase activity attained at approximately 10 d after germination (Gibson et al., 1988). For the phytase genes isolated and characterized in plants, maize (*Zea mays* L.) is the earliest plant for which phytase gene isolation has been reported (Maugenest et al., 1997, 1999). The maize phytase genes encode a homodimeric protein, with a subunit molecular mass of 38 kD, pH optimum of 4.5, and temperature optimum of 55°C. The maize coding sequence contains the amino acid sequence motif, RHGXRXP, a hallmark of histidine acid phosphatases (Ostanin et al., 1992), including fungal phytases (Ullah and Dischinger, 1993; Mitchell et al., 1997). In past several years, several other phytase genes in plant species have been isolated, such as *GmPhy* with sequence similarity to purple acid phosphatases that expressed in cotyledons of germinating soybean seedlings (Hegeman and Grabau, 2001), a Arabidopsis purple acid phosphatase *AtPAP15* with phytase activity (Kuang et al., 2009; Zhang et al., 2007), lily alkaline phytase (*LlALP1* and *LlALP2*) which possess unique catalytic properties that have the potential to be useful as feed and food supplement (Barrientos et al., 1994), and *MtPHY1*, a phytase gene sharing a high similarity to a purple acid phosphotase gene that was identified in legume model *Medicago truncatula* (Xiao et al., 2005).

Phytases have diverse potential application roles such as to be used as the animal feed supplement as well as to affect the vigor of young seedlings. In addition, as a potential phosphorus pool, the phytase and its derivatives in the arable soil could be degraded into available Pi under the hydrolysis of phytase. Over expression of several types of phytase gene such as *PhyA* of *Aspergellius niger* (Richardson et al., 2001), a synthetic phytase gene (Zimmermann et al., 2003), and *MtPHY1* (Xiao et al., 2005) in Arabidopsis all significantly improved the plant growth when the phytate was used as the sole P source. These reports suggest that part of the phytase genes have potential applications for the improvement of plant phosphorus nutrition through promotion of the utilization of organic phosphate compounds, such as the phytin reserves in the arable land. In this study, rice HAP-type phytase gene referred to *OsPHY2* was molecularly and biochemically characterized. Meanwhile, the function of this rice phytase has been elucidated based on transgene analysis. It is suggested that *OsPHY2* has a potential role in generation of elite crop germplasms with high use efficiency of phytin, a large quantity of organic phosphate compounds in the arable soil.

MATERIALS AND METHODS

Plant materials and growth

The seeds of Nipponbare (the *Japonica* subgroup) were used in this study. After surface-sterilization by 0.15% $HgCl_2$ (10 min) and thoroughly washed with ddH_2O, the seeds were put onto a wetted filter paper for germination at 28°C in darkness. The germinated seeds were then hydroponically grown in Murashige and Skoog (MS) nutrient solution under a photoperiod (12 h/12 h of day/night) at 28°C. During the 20 days growth regime, the nutrient solution was renewed regularly, generally twice in each week. For the detection of the expression profile of *OsPHY2* in the germinated seeds, the primary roots and the leaves, the whole of the seeds, roots and leaves growing at the given time points were separately sampled. Of which, the samples of the germinated seeds (covering novel primary roots, coleoptiles, and the endosperm) were collected at 5 , 10 , and 15 days after the germination. The samples of roots and leaves were harvested at 10 and 20 days after the seed germination. The samples obtained at various time points were immediately frozen in liquid nitrogen and stored at -80°C for use.

Identification and molecular characterization of *OsPHY2*

Previously, a wheat phytase gene *PhyIIc* (multiple inositol polyphosphate phosphatases, MINPP, GenBank accession number DQ995974) was functionally analyzed in our group. *In vitro* analysis suggests that PhyIIc was strongly involved in mediation of the phytate hydrolysis (data to be published). Owing to few phytase genes in rice being functional characterized to date, we searched the GenBank using the wheat *PhyIIc* as a query. As a result, a rice cDNA clone with a full-length insert (GenBank accession number NM_001058240) sharing a high similarity to the wheat query was identified. Because a rice phytase gene classified into purple acid phosphatase type identified and functionally characterized in our group and referred to *OsPHY1*, the PhyIIc homologous in rice was designated as *OsPHY2*.

The alignment analysis between *OsPHY2*, the putative rice phytase gene, and the wheat *PhyIIc* was performed based on MeAlign algorithm supplemented in DNAStar software. The isoelectric point (pI) and molecular weight (mW) of OsPHY2 were calculated using an online tool for computation of pI/Mw (http://www.expasy.org/tools/pi_tool.html). The conserved domain histidine acid phosphatase (HAP), generally identified in phytases and histidine acid phosphatases, was predicted based on conserved domain search in NCBI (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/Structure/cdd). An online algorithm (Target 1.1 server [http://www.cbs.dtu.dk/services/TargetP/]) was run for analysis of putative targeting sequences in OsPHY2. Further, an online tool (DAS, http://www.sbc.su.se/~miklos/DAS/) was adopted to predict the position of signal peptide in OsPHY2.

Identification of OsPHY2 subcellular location based on GFP fusion analysis

Binary construct pCAMBIA3301-*OsPHY2-GFP* in which the fusion *OsPHY2-GFP* was under the control of CaMV35S promoter was constructed. The complete *OsPHY2* coding sequence was amplified with 5'-CCGTCGACATGGCTGCTCCCCGCACGCC-3' and 5'-AACCATGGACAGCTCCGACTTCACATC-3', digested with *Sal*I

and *Nco*I, and ligated into the CaMV35S-*sGFP*(S65T)-Nos vector with a position in front of *GFP* (Xiao et al., 2005). The binary construct was used to transform Arabidopsis (ecotype Columbia) through the floral dip infiltration approach (Clough and Bent, 1998). The roots of the T2 plants were sampled for the detection of the subcellular location of *OsPHY2-GFP* fusion. The samples were imaged using a Leica TCS-SP5 confocal microscope (Leica Microsystems) with a 63x, numerical aperture 1.2 water-immersion objective. GFP was excited with the blue argon ion laser (488 nm), and emitted fluorescence was collected from 525 nm. The roots of the T2 Plants that transformed the free-GFP (CaMV35S-GFP) were performed to be used as the control.

Phylogenetic analysis of *OsPHY2* and its homologous

Nucleic acid sequences to be homologous of *OsPHY2* were obtained from the GenBank databases through NCBI BLAST analysis (http://www.ncbi.nlm.nih.gov). Sequences were compared within each species to eliminate incorrect or redundant entries. The phylogenetic tree was constructed based on ClustalW from Megalign 5.03 (DNASTAR, Madison, WI) by using a distance method based on comparison of 1,000 bootstrap replications.

Molecular modeling of *OsPHY2*

Based on SWISS-MODEL online tool (http://swissmodel. expasy.org/), a fully automated protein structure homology-modeling server, the theoretical three-dimensional (3D) model of OsPHY2 was predicted by using the x-ray crystallography coordinates for the known structure released in the protein database (PDB). A histidine acid phosphatase derived from the *Francisella tularensis* (FtHAP) (Singh et al., 2009), sharing a high similarity with OsPHY2 at the tertiary structure level, was identified and used for elucidation of the putative 3D model of OsPHY2. Of which, the surface charge of OsPHY2 was calculated from the constructed model, and the putative α helices and *β* sheets were manually identified based on comparisons of OsPHY2 with the subject FtHAP.

Expression analysis of OsPHY2

Total RNA of the germinated seeds, primary roots, and young leaves sampled at various time points was isolated using the TRIzol reagent (Invitrogen). The first strand of cDNA was synthesized from about 2 mg of total RNA using an M-MLV reverse transcriptase kit (TaKaRa) by following the manufacturer's suggestion. Reverse transcription polymerase chain reaction (RT-PCR) was carried out according to the descriptions of Zhang et al. (2008). Based on the *OsPHY2* cDNA sequence (GenBank accession number NM_001058240), the primers with forward and reverse orientation for specifically amplification of 3'- untranslated region (UTR) of *OsPHY2* were synthesized. The primers were 5'-AAGCTGCTACTATCCATCTACCAT'. (forward) and 5'-AAGGGCCACAATCACAACC" (reverse). The length of RT-PCR products was 266 bp. The PCR program was performed as follows: a denaturing step at 95°C for 5 min, followed by 25 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min. For validation of the RT-PCR results, each sample was conducted in three replications with independently prepared total RNA. The *OsPHY2* transcripts were normalized by *RacI*, a constitutively expressed actin gene in rice. The primers for amplification of *RacI* were '5'-CATGCTATCCCTCGTCTCGACCT" (forward) and '5'-CGCACTTCATGATGGAGTTGTAT" (reverse).

The quantitative RT (qRT)-PCR was performed on the ABI PRISM 7900HT Sequence Detection System using SYBR Green master mix (Applied Biosystems). PCR cycling was same as that used in semi-quantitative RT-PCR mentioned previously. The expression level of *RacI* was also employed as an internal standard for normalization of *OsPHY2* transcripts. For the relative quantification of gene expression, a modification of the comparative threshold cycle method was used. Relative transcript levels of the gene of interest (X) were calculated as a ratio to the *RacI* gene transcripts (U) as $(1 + E)^{-\triangle Ct}$, where $\triangle C_t$ was calculated as $(C_t^X - C_t^U)$. PCR efficiency (E) for each amplicon was calculated employing the linear regression method on the log (fluorescence) per cycle number data.

Prokaryotic expression of OsPHY2 and phytase activity analysis

For biochemical characterization of OsPHY2, the open reading frame (ORF) of *OsPHY2* (minus the 24-aa encoding sequence of the N-terminal for avoidance of the induced protein targeting to membrane) was integrated into prokaryotic expression vector pET28a (+) and the putative phytase was expressed in BL21. The ORF was amplified using the cDNAs transcribed from the 5 d-germinated seeds and the following primers: 5'-TTCCATGGCTCTCGCCGGCGGCCGAG (forward) and 5'-TTGAATTCGATGGATAGTAGCAGCTT (reverse). The PCR products were digested with *Nco*I and *Eco*RI and inserted into pET28a (+) (Novagen, Madison, WI) which was also digested by *Nco*I and *Eco*RI. The resulting plasmid, referred to pET-*OsPHY2*, was transformed into the competent *Escherichia coli* strain BL21 (DE3) (Stratagene). Cultures were grown with shaking at 37°C to midlogrithmic phase (optical density at 600 nm approximately 0.5) in Luria-Bertani medium supplemented with 50 µg/ml kanamycin, induced by adding 1 mM of IPTG, then grown an additional 12 h. The cells were harvested by centrifugation at 4,000 g for 10 min. The concurrently cultured *E. coli* strain transformed the empty vector pET28a (+) used as the control. The sonic-broken cells were used for the identification of the target protein based on SDS-PAGE analysis. The phytase activities of the sonic-broken cells were assayed according to the descriptions of Xiao et al. (2005).

Biochemical characterization of OsPHY2

For the determination of biochemical properties of OsPHY2, the activities of OsPHY2 were assayed in a range of temperatures and pH. For understanding of the effects of temperature on the enzyme activities, reactions were initiated at temperatures ranging from 27 to 77°C at 10°C intervals for 30 min at pH 3.5 with 0.5 mM phytate-Na_2 as substrate. For elucidation of the effects of pH on the enzyme activities, biochemical reactions were performed in a pH ranges from 2.5 to 6.5 at 1 pH internal for 30 min at temperature 47°C using also 0.5 mM phytate-Na_2 as substrate. To measure the effect of heat treatment on activity, the OsPHY2 were pre-incubated for 10 min at 10°C intervals from 40 to 100°C. Following pre-incubation, the enzyme samples were cooled to room temperature and the enzyme activities were assayed same under a temperature of 47°C and a pH of 3.5 supplemented with the substrate phytate-Na_2 (in 50 mM NaOAc, pH 4.5). The phytase activities were assayed according to the descriptions of Xiao et al. (2005).

Construction of a chimeric binary construct fused potato signal peptide sequence (PSP) and the open reading frame of *OsPHY2*

A chimeric binary plasmid fused the potato patatin signal peptide (PSP) and the open reading frame (ORF) of *OsPHY2*. The PSP, previously confirmed to possess the ability to direct the polypeptide into the root-soil interface (Xiao et al., 2006), was PCR amplified using the potato DNA and specific primers and inserted in front of

the open reading frame of *OsPHY2* without the frame shift. For avoidance of the possible interference of the 24-aa signal peptide, the sequence encoding the signal peptide of *OsPHY2* was not included in the *PSP-OsPHY2* fusion. The primers for amplification of *OsPHY2* ORF were 5'- TTCCATGGCTCTCGCCGGCGGCCGAG (forward) and 5'- TTGGTAACCGATGGATAGTAGCAGCTT (reverse). The *PCR* products were double digested by *Nco*I and *BstE*II, and then were integrated into binary expression vector pCAMBIA3301 that were also double digested by the previous enzymes. The *PSP* was amplified using the potato genome DNA and following primers: "5'-TTCCATGGCAACTACTAAATCTTTT" (forward) and 5'-TTCCATGGGCGTAGCACATGTTGAACT (reverse). After digested by *Nco*I, the PCR products were integrated into the *OsPHY2* ORF integrated binary plasmids with the position being in front of the *OsPHY2* ORF. After sequencing confirmation, the chimeric construct was transferred into *Agrobacterium tumefaciens* (strain EHA105) and genetic transformation of tobacco was performed by following the descriptions of Guo et al. (2009).

Measurements of phytase activities, contents of Pi and the total P in the transgenic plants that were grown under phytate-Na_2 as sole P source

The generated transgenic T1 plants were grown in a growth room to maturity. The T2 transgenic lines generated from thirteen independent transformation events with single target gene integrated were subjected to analysis of the target gene transcripts based on qRT-PCR. The qRT-PCR was performed same as in detection of *OsPHY2* transcripts in the germinated seeds, roots, and leaves mentioned previously except the primers used: 5'-CTCTCGCCGGCGGCCGAGACCG (forward) and 5'-TACAGCTCCGACTTCACATCCT (reverse). Based on the detection of the *OsPHY2* transcripts in the control (CK, transformed the empty vector) and the transgenic lines, two lines with strong *OsPHY2* expression (Line 5 and Line 9), one line with weak OsPHY2 expression (Line 3), and CK were selected for further analysis. For that the seeds from the transgenic lines and the control were separately grown in a plastic tray filled with well mixed medium (vermiculite containing 1% phytate-Na_2, w/w) for three weeks. During the growth, the plants were regularly supplemented with MS (without Pi) solution. After the treatment, the phytase activities, total P, and the dry weight of the transgenic plants and the control were analyzed.

The phytase activities of the transgenic plants and the control were assayed according to the descriptions of Xiao et al. (2005). The contents of Pi and total P were measured by following the regular molybdenum-blue assay method. The dry weight of the control and transgenic lines were obtained by drying three representative plants in an oven under 90°C for 24 h.

RESULTS

Identification of *OsPHY2*, a phytase gene that classified into HAP-type in rice

Using the sequence of wheat phytase gene *PhyIIc* (multiple inositol polyphosphate phosphatases, MINPP, GenBank accession number DQ995974) as a query, a cDNA clone with a full-length insert (GenBank accession number NM_001058240) sharing a high similarity to the wheat MINPP was identified based on BLAST search in NCBI website (http://www.ncbi.nlm.nih.gov/). Because the uncharacterized rice cDNA shares a high similarity to wheat phytase gene, and another phytase gene in rice classified into purple acid phosphotase type, we have designated *OsPHY1* previously (unpublished data), this putative rice phytase gene was referred to *OsPHY2* hereafter. The identity between *OsPHY2* and the wheat *PhyIIc* is 77.2% at the nucleic acid level. The alignment results of these two phytase genes are shown in Figure 1.

Molecular characterization of *OsPHY2*

OsPHY2 has a cDNA of 2040 bp in full-length, with an open reading frame of 1560 bp and encoding a 519-aa polypeptide (Figure 2). OsPHY2 has a predicted molecular weight of 57.99 kD and an isoelectric point of 7.59. In the meantime, OsPHY2 contains a histidine phosphatase domain (residue 48 to 441) that is generally conserved in histidine acid phosphatase (HAP)-type phytases. Similar to the histidine acid phosphatases (HAPs) identified in fungi and angiosperms (Ostanin et al., 1992; Ullah and Dischinger, 1993; Mitchell et al., 1997), OsPHY2 contains the amino acid sequence motif, RHGXRXP (residue 77 to 83), acts as a hallmark in the HAPs (Figure 2). However, OsPHY2 does not share high similarities to the maize phytase genes, and lacks any additional regions of high sequence homology to fungal phytases, although, all of them have the motif RHGXRXP. These results suggest that *OsPHY2* and other phytase genes identified in maize and fungi derived from different ancestors.

OsPHY2 was targeted to the cytoplasm membrane

Analysis of putative targeting sequences using an online algorithm (Target 1.1 server [http://www.cbs.dtu.dk/services/TargetP/]) suggests that OsPHY2 is targeted to the cytoplasm membrane after ER sorted, with a coefficient of 0.848 assigned to signal peptide (SP), which is much higher than coefficients of 0.033, 0.037 and 0.004 assigned to chloroplast transit peptide (cTP), mitochondrial targeting peptide (mTP), and other subcellular signaling, respectively. For predication of the SP length in OsPHY*2*, another online tool (DAS, http://www.sbc.su.se/~miklos/DAS/), a transmenbrane predication server, was adopted. The SP was located at the residue 1 to 24 at the N-terminal. Further, to experimentally determine the subcellular localization of OsPHY2, the open reading frame of *OsPHY2* (minus the translation start codon TAG) was inserted in front of a green fluorescent protein (GFP) reporter gene. In the roots of the *OsPHY2-GFP* fusion transgenic Arabidopsis, it is clearly observed that the fusion was targeted on top of the cytoplasm membrane (Figure 3). Therefore, OsPHY2 is located at the cytoplasm membrane owing to the guidance of the 24-aa signal peptide at the N-terminal.

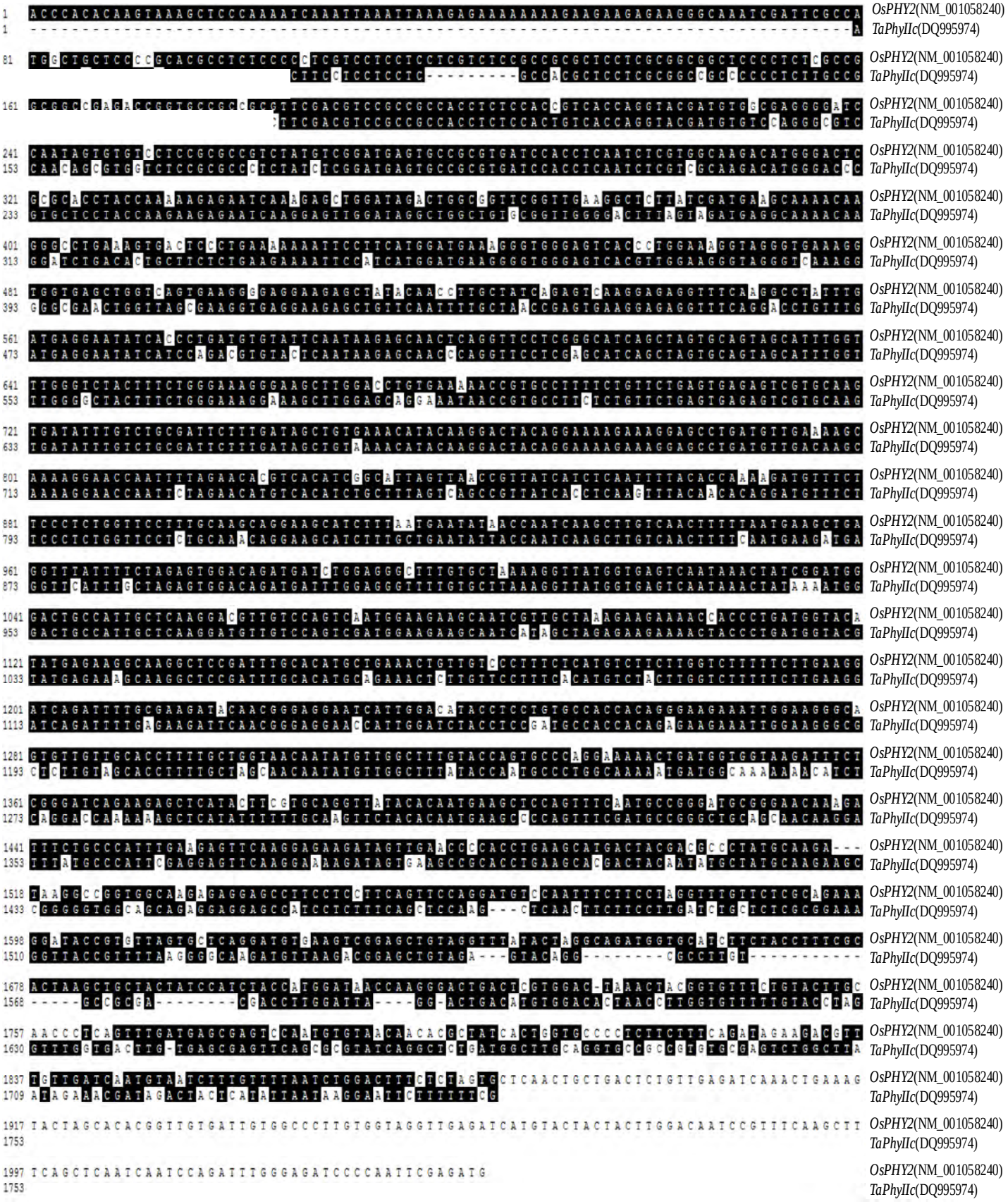

Figure 1. Alignment analysis of *OsPHY2* and a wheat phytase gene *TaPhyIIc*.

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   1  ACCCACACAAGTAAAGCTCCCAAAATCAAATTAAATTAAAGAGAAAAAAAAGAAGAAGAGAAGGGCAAATCGATTCGCCATGGCTGCT
                                                                                  M  A  A
  89  CCCCGCACGCCTCTCCCCCTCGTCCTCCTCCTCGTCTCCGCCGCGCTCCTCGCGGCGGCTCCCCTCTCGCCGGCGGCCGAGACCGGTGCC
       P  R  T  P  L  P  L  V  L  L  L  V  S  A  A  L  L  A  A  A  P  L  S  P  A  A  E  T  G  A
 179  GCCGCGTTCGACGTCCGCCGCCACCTCTCCACCGTCACCAGGTACGATGTGGCGAGGGGATCCAATAGTGTGTCCTCCGCGCCGTCTATG
       A  A  F  D  V  R  R  H  L  S  T  V  T  R  Y  D  V  A  R  G  S  N  S  V  S  S  A  P  S  M
 269  TCGGATGAGTGCCGCGTGATCCACCTCAATCTCGTGGCAAGACATGGGACTCGCGCACCTACCAAAAAGAGAATCAAAGAGCTGGATAGA
       S  D  E  C  R  V  I  H  L  N  L  V  A  R  H  G  T  R  A  P  T  K  K  R  I  K  E  L  D  R
 359  CTGGCGGTTCGGTTGAAGGCTCTTATCGATGAAGCAAAACAAGGGCCTGAAAGTGACTCCCTGAAAAAAATTCCTTCATGGATGAAAGGG
       L  A  V  R  L  K  A  L  I  D  E  A  K  Q  G  P  E  S  D  S  L  K  K  I  P  S  W  M  K  G
 449  TGGGAGTCACCCTGGAAAGGTAGGGTGAAAGGTGGTGAGCTGGTCAGTGAAGGGGAGGAAGAGCTATACAACCTTGCTATCAGAGTCAAG
       W  E  S  P  W  K  G  R  V  K  G  G  E  L  V  S  E  G  E  E  E  L  Y  N  L  A  I  R  V  K
 539  GAGAGGTTTCAAGGCCTATTTGATGAGGAATATCACCCTGATGTGTATTCAATAAGAGCAACTCAGGTTCCTCGGGCATCAGCTAGTGCA
       E  R  F  Q  G  L  F  D  E  E  Y  H  P  D  V  Y  S  I  R  A  T  Q  V  P  R  A  S  A  S  A
 629  GTAGCATTTGGTTTGGGTCTACTTTCTGGGAAAGGGAAGCTTGGACCTGTGAAAAACCGTGCCTTTTCTGTTCTGAGTGAGAGTCGTGCA
       V  A  F  G  L  G  L  L  S  G  K  G  K  L  G  P  V  K  N  R  A  F  S  V  L  S  E  S  R  A
 719  AGTGATATTTGTCTGCGATTCTTTGATAGCTGTGAAACATACAAGGACTACAGGAAAAGAAAGGAGCCTGATGTTGAAAAGCAAAAGGAA
       S  D  I  C  L  R  F  F  D  S  C  E  T  Y  K  D  Y  R  K  R  K  E  P  D  V  E  K  Q  K  E
 809  CCAATTTTAGAACACGTCACATCGGCATTAGTTAACCGTTATCATCTCAATTTTACACCAAAAGATGTTTCTTCCCTCTGGTTCCTTTGC
       P  I  L  E  H  V  T  S  A  L  V  N  R  Y  H  L  N  F  T  P  K  D  V  S  S  L  W  F  L  C
 899  AAGCAGGAAGCATCTTTAATGAATATAACCAATCAAGCTTGTCAACTTTTTAATGAAGCTGAGGTTTATTTTCTAGAGTGGACAGATGAT
       K  Q  E  A  S  L  M  N  I  T  N  Q  A  C  Q  L  F  N  E  A  E  V  Y  F  L  E  W  T  D  D
 989  CTGGAGGCTTTGTGCTAAAAGGTTATGGTGAGTCAATAAACTATCGGATGGGACTGCCATTGCTCAAGGACGTTGTCCAGTCAATGGAA
       L  E  G  F  V  L  K  G  Y  G  E  S  I  N  Y  R  M  G  L  P  L  L  K  D  V  V  Q  S  M  E
1079  GAAGCAATCGTTGCTAAAGAAGAAAACCACCCTGATGGTACATATGAGAAGGCAAGGCTCCGATTTGCACATGCTGAAACTGTTGTCCCT
       E  A  I  V  A  K  E  E  N  H  P  D  G  T  Y  E  K  A  R  L  R  F  A  H  A  E  T  V  V  P
1169  TTCTCATGTCTTCTTGGTCTTTTTCTTGAAGGATCAGATTTTGCGAAGATACAACGGGAGGAATCATTGGACATACCTCCTGTGCCACCA
       F  S  C  L  L  G  L  F  L  E  G  S  D  F  A  K  I  Q  R  E  E  S  L  D  I  P  P  V  P  P
1259  CAGGGAAGAAATTGGAAGGGCAGTGTTGTTGCACCTTTTGCTGGTAACAATATGTTGGCTTTGTACCAGTGCCCAGGAAAAACTGATGGT
       Q  G  R  N  W  K  G  S  V  V  A  P  F  A  G  N  N  M  L  A  L  Y  Q  C  P  G  K  T  D  G
1349  GGTAAGATTTCTCGGGATCAGAAGAGCTCATACTTCGTGCAGGTTATACACAATGAAGCTCCAGTTTCAATGCCGGGATGCGGGAACAAA
       G  K  I  S  R  D  Q  K  S  S  Y  F  V  Q  V  I  H  N  E  A  P  V  S  M  P  G  C  G  N  K
1439  GATTTCTGCCCATTTGAAGAGTTCAAGGAGAAGATAGTTGAACCCCACCTGAAGCATGACTACGACGCCCTATGCAAGATAAGGCCGGTG
       D  F  C  P  F  E  E  F  K  E  K  I  V  E  P  H  L  K  H  D  Y  D  A  L  C  K  I  R  P  V
1529  GCAAGAGAGGAGCCTTCCTCCTTCAGTTCCAGGATGTCCAATTTCTTCCTAGGTTTGTTCTCGCAGAAAGGATACCGTGTTAGTGCTCAG
       A  R  E  E  P  S  S  F  S  S  R  M  S  N  F  F  L  G  L  F  S  Q  K  G  Y  R  V  S  A  Q
1619  GATGTGAAGTCGGAGCTGTAGGTTTATACTAGGCAGATGGTGCATCTTCTACCTTTCGCACTAAGCTGCTACTATCCATCTACCATGGAT
       D  V  K  S  E  L
1709  AACCAAGGGACTGACTCGTGGACTAAACTACGGTGTTTCTGTACTTGCAACCCTCAGTTTGATGAGCGAGTCCAATGTGTAACAACACGC
1799  TATCACTGGTGCCCCTCTTCTTTCAGATAGAAGACGTTTGTTGATCAATGTAATCTTTGTTTTAATCTGGACTTTCTCTAGTGCTCAACT
1889  GCTGACTCTGTTGAGATCAAACTGAAAGTACTAGCACACGGTTGTGATTGTGGCCCTTGTGGTAGGTTGAGATCATGTACTACTACTTGG
1979  ACAATCCGTTTCAAGCTTTCAGCTCAATCAATCCAGATTTGGGAGATCCCCAATTCGAGATG
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Figure 2. The cDNA sequence of *OsPHY2* and the corresponding translated polypeptide. The conserved amino acid sequence motif, RHGXRXP (residue 77 to 83) that acts as a hallmark in OsPHY2 is shadow labeled. The putative signal peptide locating at the N-terminal (residue 1 to 24) is underline labeled.

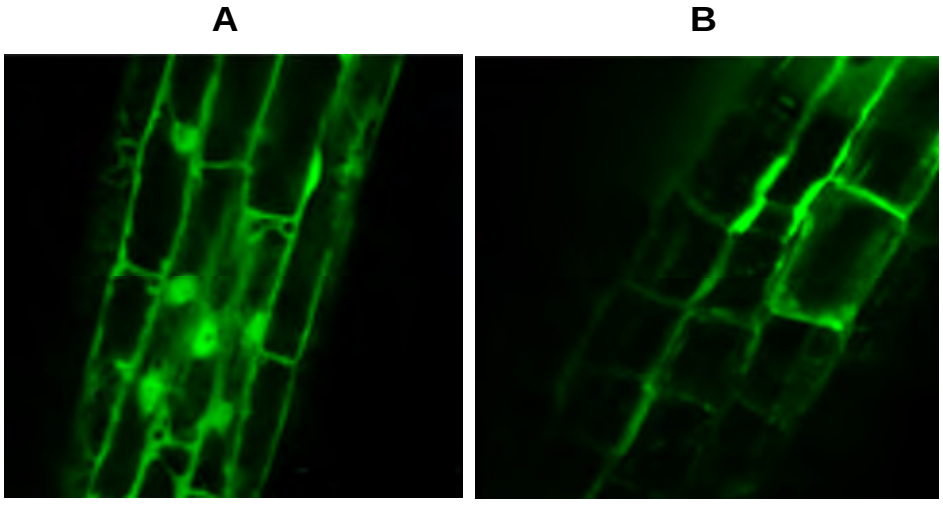

Figure 3. Subcellular location of OsPHY2 based on confocal laser scanning microscopy analysis. (A) The GFP fluorescence was detected in the Arabidopsis plant transformed the empty binary plasmid. (B) The GFP fluorescence was detected in the Arabidopsis plant transformed the binary plasmid that fused *OsPHY2-GFP*. The roots of the plants were sampled and subjected to the signal scanning analysis.

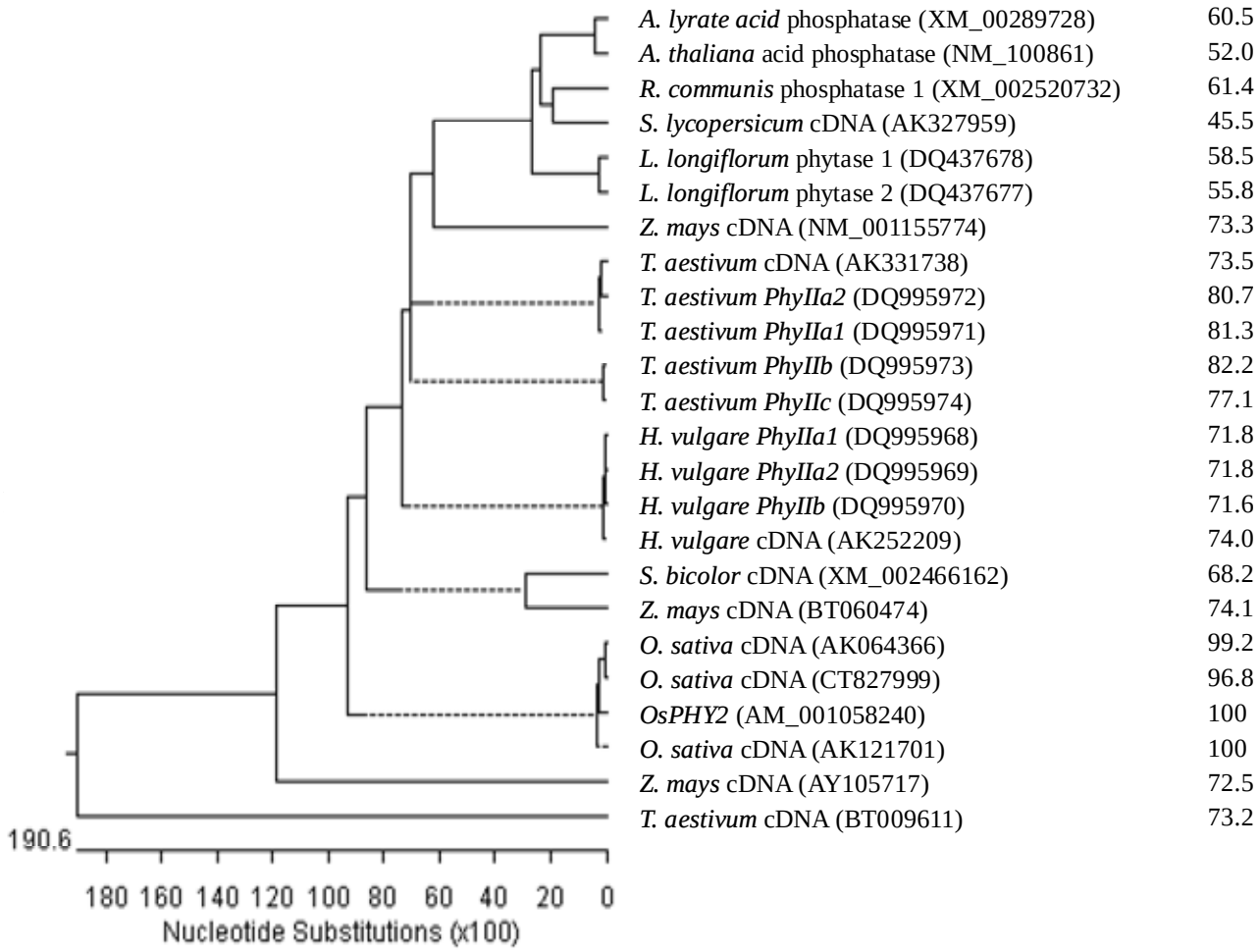

Figure 4. Phylogenetic relationships among *OsPHY2* and its homologous in plant species.

Phylogenetic analysis of *OsPHY2*

OsPHY2 was used as a query sequence in a BLASTX search for similarities to other sequences in GenBank. Among the identified homologous of *OsPHY2*, no homology was revealed to any of the previously reported phytase sequences from maize or microbes. Phylogenetic analysis suggests that *OsPHY2* and its plant homologous could be classified into four groups, including group I to group IV. The top scoring results against to *OsPHY2* included the multiple inositol polyphosphate phosphatase genes released in wheat (*Triticum aestivum* L.) and barley (*Hordeum vulgare* L.) (sharing similarities of 71.6% to 82.2), as well as the phyatse genes in *L. longiflorum*, acid phosphatase genes in *Arabidopsis thaliana*, and the cDNAs from diverse plants with uncharacterized functions (Figure 4).

These results implicate that *OsPHY2* derived a different progenitor from its plant homologous.

Molecular modeling of OsPHY2

Based on SWISS-MODEL (http://swissmodel.expasy.org/), a fully automated protein structure homology-modeling server, the theoretical three-dimensional (3D) model of OsPHY2 was derived by using the x-ray crystallography coordinates for the known structure of histidine acid phosphatase, a homologous from the *Francisella tularensis* (FtHAP) (Singh et al., 2009). The surface charge of OsPHY2 was calculated from the constructed model, indicating the presence of considerable surface areas with positive charge (blue areas in Figure 5), as expected for a basic protein and to be conserved in the histidine acid phosphatses. In addition, despite the relative high sequence divergence between OsPHY2 and

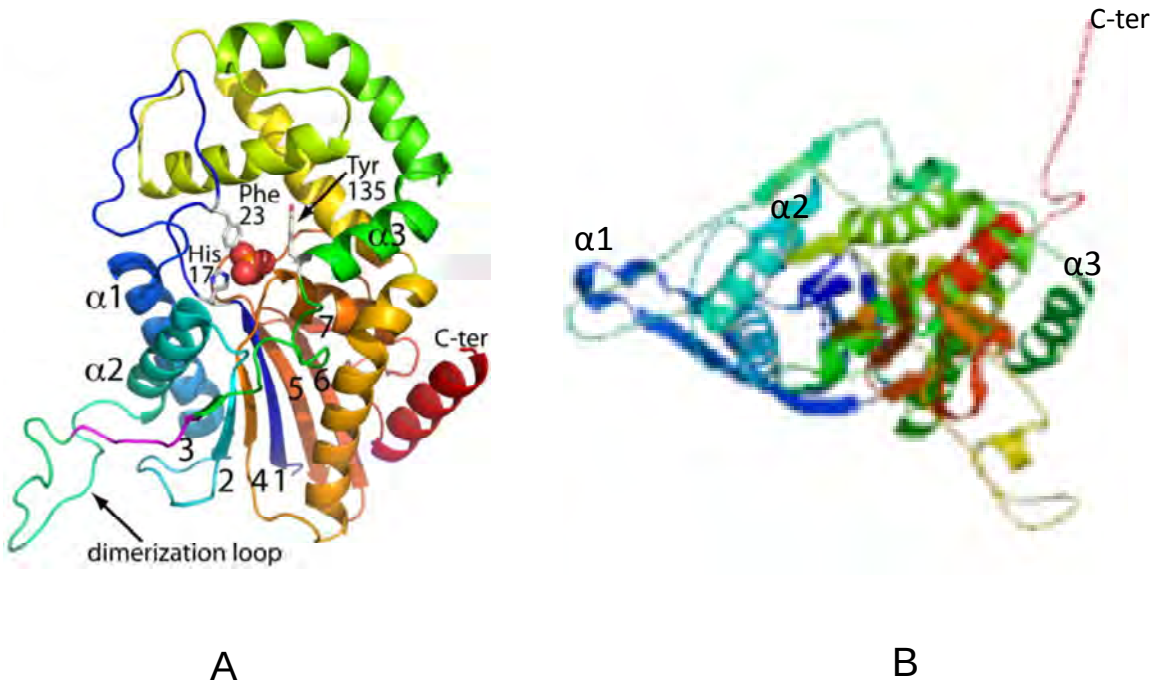

Figure 5. Structural representations of molecular models of OsPHY2 and PtHAP, a histidine acid phosphatse sharing a high similarity to OsPHY2 in *Francisella tularensis* (A) The theoretical three-dimensional (3D) model of PtHAP. (B) The theoretical three-dimensional (3D) model of OsPHY2.

PtHAP, overall predicted enzyme structures are fairly similar, showing that OsPHY2 contains three similar α helices (α1, α2, and α3), and several similar *β* sheets. In the meantime, similar to the structure of the FtHAP determined at 1.50 A resolution, OsPHY2 exhibits a two-domain fold that is composed of an alpha/beta core domain and a smaller domain that caps the core domain. The structures show that the core domain supplies the phosphoryl binding site, catalytic histidine (putative His41, whereas the FtHAP being His17), and an aspartic acid residue (putative Asp302, whereas FtHAP being Asp261) that protonates the leaving group with the cap domain to contribute residues that enforce substrate preference. These predicted structural data of OsPHY2 are consistent with a role for the rice phytase in scavenging phosphate from phytins present in the seeds.

Expression patterns of *OsPHY2* in the germinated seeds, roots and leaves

The expression patterns of *OsPHY2* in the germinated seeds, the primary roots and the leaves in the young seedlings were investigated based on semi-quantitative reverse transcription (RT)-PCR and qRT-PCR analysis. Of the tested tissues, the most abundant transcripts of *OsPHY2* were detected in the germinated seeds, with a trend to be gradually elevated along with the seed germination progression (Figure 6A and B). The expression levels of *OsPHY2* in the roots were kept stable and were much lower than those of the germinated seeds. The lowest transcript levels of *OsPHY2* were detected in the leaves, showing a pattern to be dropped along the growth progress (Figure 6A and B). The much more transcripts of *OsPHY2* were observed in the germinated seeds suggesting that OsPHY2 is possibly involved in the hydrolysis of seed phytins and plays major role in the supply of the Pi for the rapid cell growth and division during the seed germination.

Characteristics of the prokaryotic expressed OsPHY2

For the determination of the biochemical characteristics of OsPHY2, the open reading frame (ORF) of *OsPHY2* was integrated into a prokaryotic expression vector pET28a(+) and expressed in the *E. coli* host BL21. The induced target protein is shown in Figure 7A. The prokaryotic expressed OsPHY2 was subjected to a range of temperature and pH and the phytase activities were assayed. In a range of tested temperatures from 27 to 77°C, the highest enzymatic activity of OsPHY2 was reached at 47°C (Figure 7B). Similarly, in a range of tested pH from 2.5 to 6.5, the highest enzymatic activity of OsPHY2 was obtained at pH 3.5 (Figure 7C). In the meantime, the thermostability of the induced OsPHY2 was also tested by conducting a 10 min pre-incubation at temperatures ranging from of 40 to 100°C prior to the standard activity assay (Figure 7D). The results indicate that OsPHY2 possesses relative strong enzyme activities

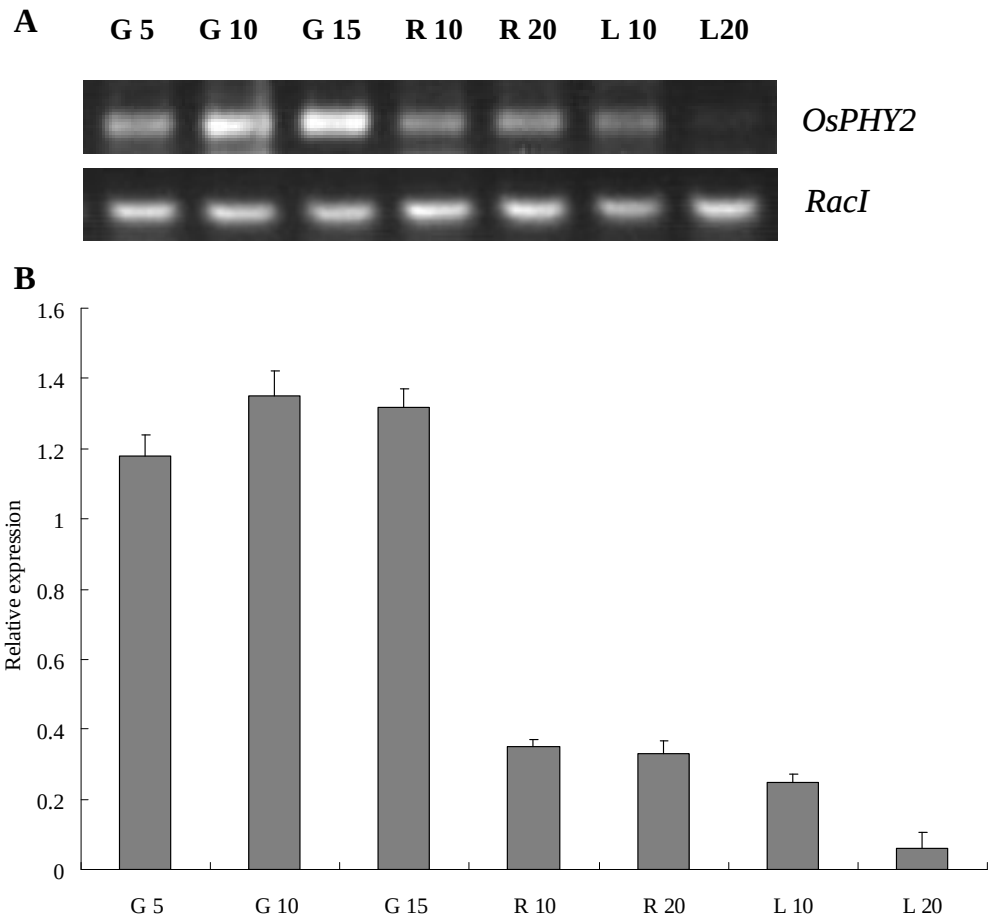

Figure 6. The transcripts of *OsPHY2* in the germinated seeds, primary roots, and young leaves. (A) Results of semi-quantitative RT-PCR analysis. (B) Results of qRT-PCR analysis. G 5, G 10, and G 15 represent the germinated seed samples of 5 d, 10 d, and 15 d after seed germination, respectively. R 10 and R 20 represent the primary root samples of 10 d and 20 d after seed germination, respectively. L 10 and L 20 represent the leaf samples of 10 d and 20 d after seed germination, respectively.

up to 80°C, but does not regain activity after heat denaturation at temperatures over 80°C.

Over expression of *OsPHY2* in tobacco significantly improved plant growth when phytate act as the sole P source in the medium

A binary plasmid integrated with a fusion of the potato signal peptide (SP) and *OsPHY2* open reading frame (*SP-OsPHY2*) was constructed and the schematic diagram is represented in Figure 8A. Thirteen independent transgenic T2 tobacco plants that integrated just one copy of the fusion were identified and subjected to identification of the target gene transcripts, of which the expression levels of *OsPHY2* were varied largely (Figure 8B). For determination of the role of OsPHY2 on mediation of the phytate hydrolysis in the growth medium, two lines with strong *OsPHY2* expression (Line 5 and Line 9), one line with weak *OsPHY2* expression (Line 3), and the control (CK) were selected for further analysis. After 3-week growth under the condition that phytate-Na_2 was the sole P source, dramatically variations on the plant phenotypes among the transgenic lines and CK could be observed, showing that *OsPHY2*-overexpressing transgenic lines displayed a pronounced improvement on the plant growth. The lines with strong *OsPHY2* expression showed to grow much better than the line with weak *OsPHY2* expression (Figure 8C). These results suggest that the overexpressed OsPHY2 in tobacco could be secreted into the rhizhosphere and plays an important role on degradation of the medium phytate.

Further, the phytase activities, total P contents, accumulative P amount per plant, and plant dry weight of the transgenic lines and CK were investigated after 3 weeks growth under the condition of phytate-Na_2 as sole P source. In accordance with the *OsPHY2* expression levels and the plant phenotype displayed after 3 weeks

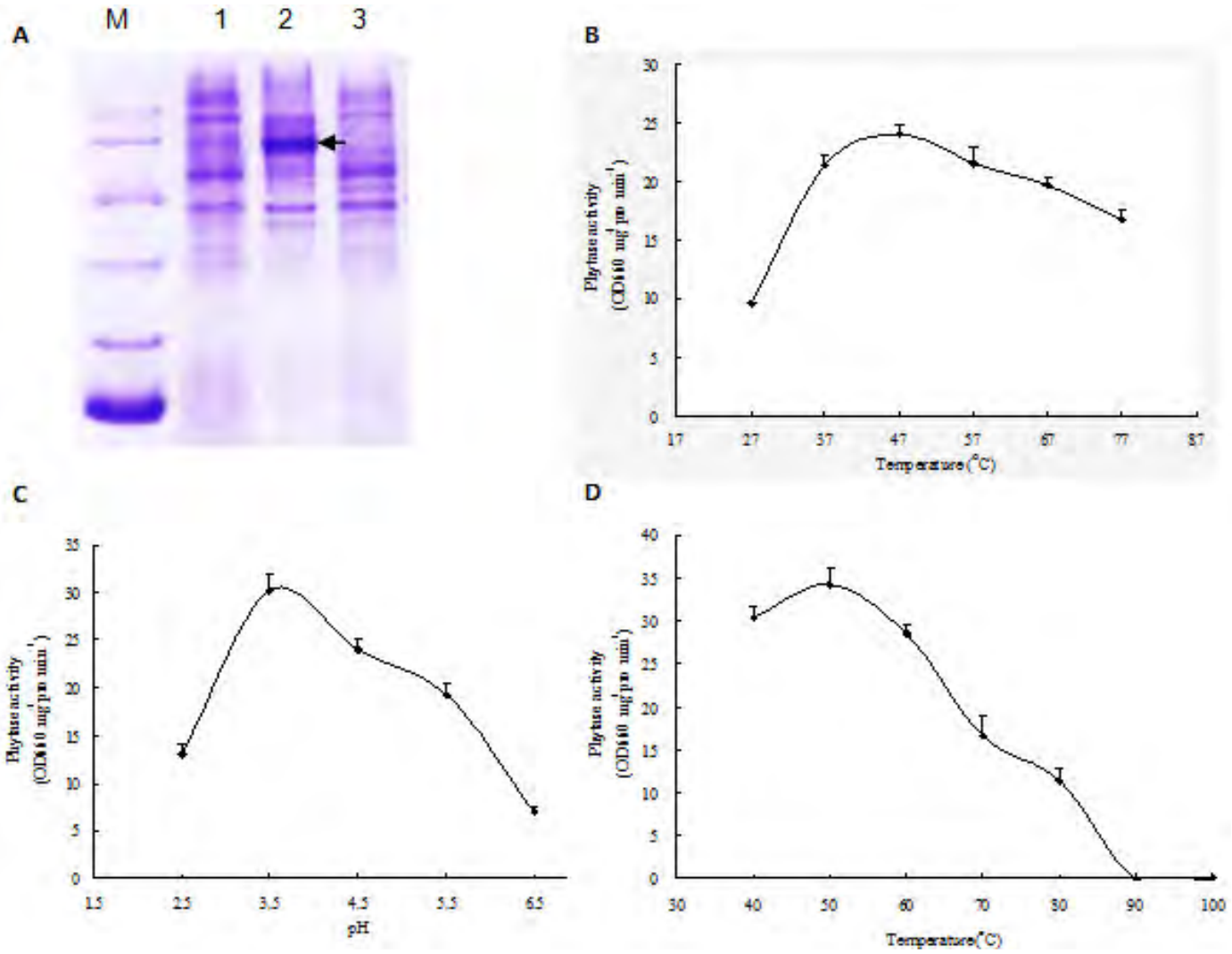

Figure 7. Effects of temperature and pH on the phytase activity of OsPHY2. (A) The SDS-PAGE analysis showing that *OsPHY2* was induced in *E. coli* strain. M: Protein standard ladder, Lane 1: *E. coli* strain transformed empty expression vector; Lane 2: *E. coli* transformed expression plasmid harboring the ORF of *OsPHY2* after 6 h of IPTG induced; Lane 3: *E. coli* transformed expression plasmid harboring the ORF of *OsPHY2* without IPTG induced. The arrow points to the induced OsPHY2. (B)The phytase activities of OsPHY2 assayed in temperatures from 27 to 77°C. (C) The phytase activities of OsPHY2 assayed from pH 2.5 to 6.5. (D) The thermostabilities of OsPHY2 determined by preincubating samples at temperatures ranging from 40 to 100°C for 10 min and subsequently assaying remaining phytase activity.

treatment, the lines with strong expression of *OsPHY2* (Line 5 and Line 9) also showed higher phytase activities and total P contents, and much more of plant dry mass and accumulative P amount (Figure 9A to D). These results clearly demonstrate that OsPHY2 is involved in the hydrolysis of the medium phytate and play an important role on the improvement of plant growth by alleviation of the P stress when the phytate was used as the sole P source.

DISCUSSION

Myo-inositol hexakisphosphate ($InsP_6$; phytic acid) is the most abundant form of phosphorus found in plant seeds. In the cereal seed, the $InsP_6$ is bound to minerals (K, Ca, Mg, Zn and Mn) to form inclusion particles (globoids) which are commonly located in protein storage vacuoles (Lott et al., 1979). During the seed germination, $InsP_6$ is hydrolysed into *myo*-inositol and inorganic phosphates (Pi) by phytase (*myo*-inositol hexakisphosphate phosphatase) and used for subsequent seedling growth (Loewus and Murthy, 2000; Raboy, 2003). Thus far, totally four distinct classes of phytase have been characterized in the literature, including HAPs, B-propeller phytases, purple acid phosphatases (Mullaney et al., 2003), and most recently, protein tyrosine phosphatase-like phytases (PTP-like phytases) (Puhl et al., 2007). Of which, most of the known phytases belong to a class of enzyme called HAPs. HAPs have been isolated from diverse organisms

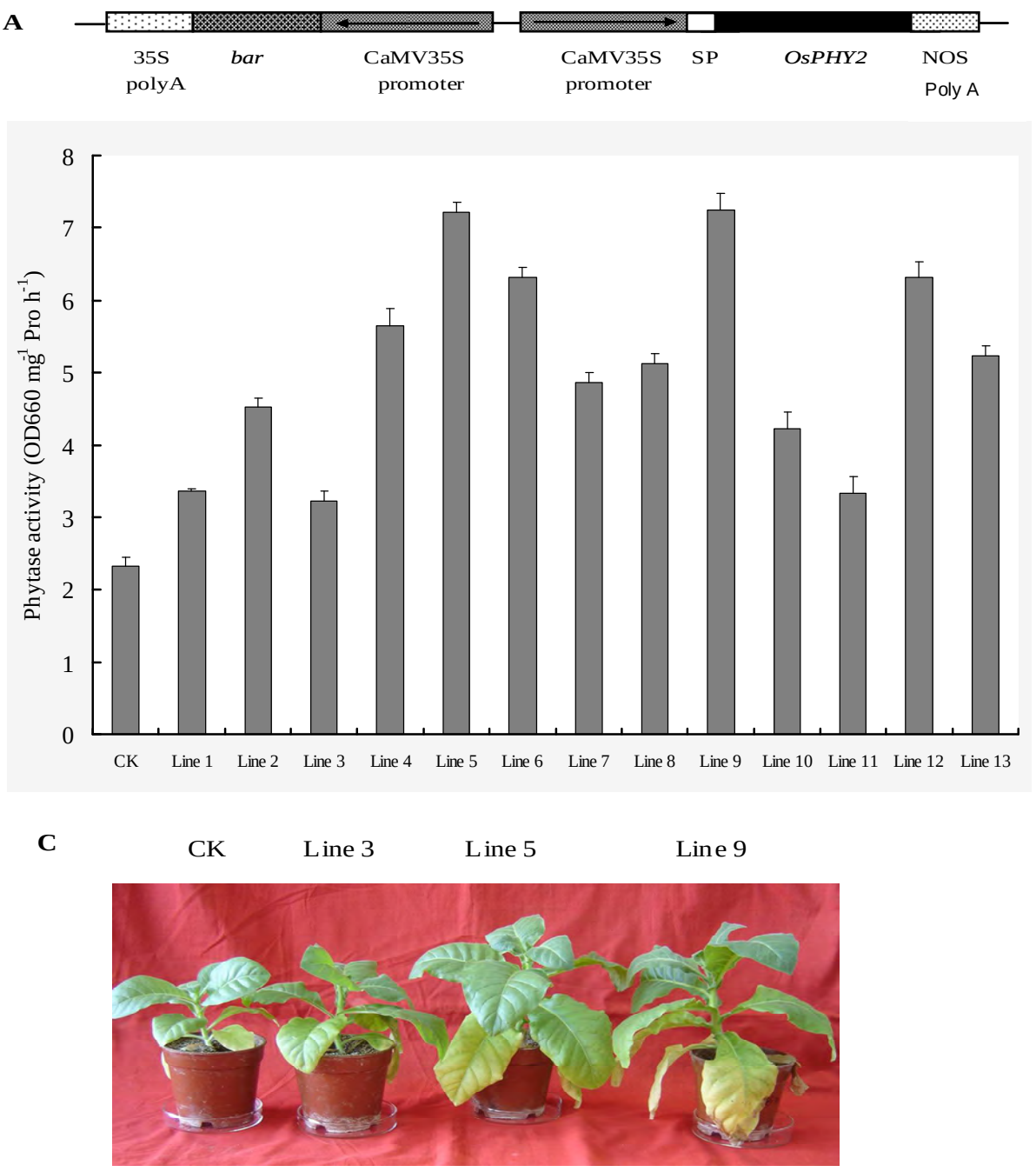

Figure 8. The transcripts of *OsPHY2* in the generated transgenic tobacco plants and plant phenotypes of the typical transgenic lines when subjected to growth under phytate to be sole P source. (A) A schematic diagram of the constructed binary plasmid harboring *OsPHY2*. (B) The phytase activities in the generated transgenic tobacco plants. (C) The plant phenotypes of three typical transgenic lines and CK when subjected to growth under phytate to be sole P source.

such as filamentous fungi, bacteria, yeast, and plants (Mullaney et al., 2000). It is noted that all members of this class of phytase share a common active site sequence motif (Arg-His-Gly-X-Arg-X-Pro) and hydrolyzes phytic acid and its derivatives with a two-step mechanism manner (Mullaney et al., 2003). In this study, a rice phytase gene referred to *OsPHY2* sharing a high similarity to wheat histidine acid phospahtase *PhyIIc* was molecularly and functionally characterized. It was found that OsPHY2 possess the typical molecular characterization including to contain a conserved active sequence motif AHGXAP (Ullah and Dischinger, 1993;

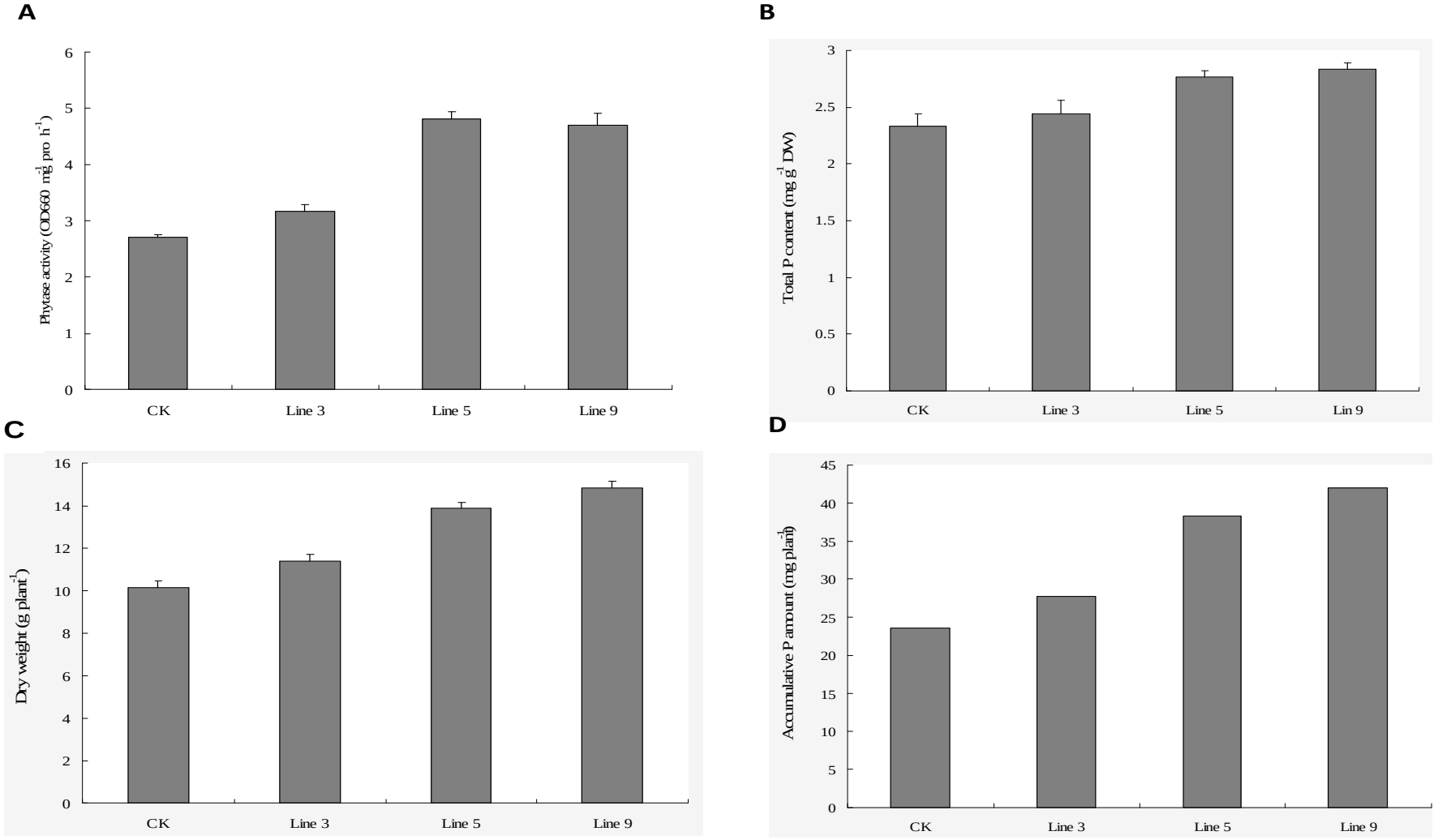

Figure 9. The phytase activities, total P contents, and plant dry weights and accumulative P amounts in control (transformed empty vector) and the transgenic tobacco lines ectopically expressing OsPHY2 (A) phytase activities, (B) total P contents, (C) plant dry weights, (D) accumulative amounts per plant. The plants were grown 3-week under the condition of phytate-Na_2 as sole P source..

Maugenest et al., 1999), a huge HAP domain, and a conserved phosphoryl binding site which is composed of a conserved catalytic histidine (putative His41) and an aspartic acid residue (putative Asp302). Phylogenetic analysis displays that *OsPHY2* shares high similarity to HAPs in

wheat and barley. However, no similarities were detected between *OsPHY2* with the *HAPs* derived from maize, the early phytases identified in plant species (Maugenest et al., 1997, 1999), and fungi. These results have indicated that *OsPHY2* and its homologous such as those identified in wheat and barley were derived from different ancestors in contrast to the HAPs in maize and fungi.

In this study, green fluorescent protein (GFP) reporter gene was used to examine the subcellular localization of OsPHY2 after sorted through endoplasmic reticulum system. Under the control of CaMV35S promoter, the fused *OsPHY2* and *GFP* gene were expressed in Arabidopsis constitutively and those expressed in roots were subjected to detection of the *GFP* signal. It is observed that the fluorescence was confined to the cytoplasm membrane, in strikingly contrast to the signals detected were freely distributed in every positions of the cell in the control that transformed the sole *GFP* gene. The subcellular location determined by the fusion gene was consistent with the results of targeting prediction analysis based on Target1.1 and DAS. Therefore, OsPHY2 is targeted to the cytoplasm membrane.

For understanding of the theoretical three-dimensional (3D) model of OsPHY2, SWISS-MODEL on line tool (http://swissmodel.expasy.org/), a fully automated protein structure homology-modeling server, was used to define the putative tertiary structure of this rice phytase. As a result, a histidine acid phosphatase from the FtHAP released in the PDB (Singh et al., 2009) was identified to be used as the structural model of OsPHY2. It is observed that the considerable surface area of OsPHY2 was positively charged, as expected for a basic protein and to be conserved in the HAPs. In the meantime, OsPHY2 contains three α helices (α1, α2, and α3) and *β* sheets similar to FtHAP, exhibiting a two-domain fold that is composed of an alpha/beta core domain and a smaller domain that caps the core domain. These predicted structural data suggest that OsPHY2 possess the molecular characterizations of histidine acid phosphatase and is possibly functional as a phytase gene in scavenging phosphate from phytins present in the seeds.

Animal feeds are comprised primarily of plant seed components, typically from corn and soybean. However, seed phytic acid is largely unavailable to monogastric animals, including poultry, swine, fish, and humans (Reddy et al., 1989; Ravindran et al., 1995). The excretion of undigested phytic acid in manure leads to the redistribution of phosphorus to the soil. An undesirable side effect of high soil phosphorus levels is the loss of this important nutrient, due to its entry into watersheds through runoff. Furthermore, as a limiting nutrient in aquatic environments, elevated phosphorus levels can lead to eutrophication and water quality issues (Sharpley et al., 1994). Therefore, phytases have potential applications to be used as the seed supplements as well as to exert positive affections on the environment. In past two decades, the HAP type phytase from the fungus *Aspergillus niger* is well known for its high specific activity and its commercially marketed role as an animal feed additive to increase the bioavailability of phosphate from phytic acid in the grain-based diets of poultry and swine (Kim et al., 2006). HAPs have also been over expressed in several transgenic plants as a potential alternative method of phytase production for the animal feed industry (Chen et al., 2006). In this study, the prokaryotic expressed OsPHY2 was confirmed to exhibit strong enzyme activities, with an optimal temperature of 47°C and a suitable pH of 3.5 in the biochemical reactions. These biochemical properties have been implicated that OsPHY2, a phytase gene derived from rice, could be acted as a useful potential target gene on commercially production of phytases used for animal feed additive.

Phosphorus (P) is a critical macronutrient for plant growth and development. Terrestrial plants generally take up soil P in its inorganic form (Marschner, 1995). However, 50 to 80% of the total P in arable lands exists as organic phosphate, in which, up to 60 to 80% is phytate (myoinositol hexakisphosphate; Iyamuremye et al., 1996; Turner et al., 2002; George and Richardson, 2008). Since phytate-P is not directly available to plants, low P availability becomes one of the limiting factors to plant growth. As a special type of APases, phytases possess a capability to hydrolyze phytate and its derivatives and to have demonstrated to be important for utilizing Pi from phytate in the growth medium (Asmar, 1997; Li et al., 1997; Hayes et al., 1999; Richardson et al., 2000). Thus far, some success has been obtained in engineering plants that acquire Pi more efficiently through ectopic expression of secreted phytases (Richardson et al., 2001; Zimmermann et al., 2003; Xiao et al., 2005). In this study, the rice *OsPHY2* gene directed by an extracellular targeting sequence from a potato patatin gene was transformed into tobacco plants. Under the condition that phyate was used as the sole P source, the transgenic plants over expressing *OsPHY2* showed higher phytase activities and total P contents, and much more of plant dry mass and accumulative P amount (Figure 9A to D) as well as much more improved plant growth (Figure 8C). These results clearly confirm that OsPHY2 is involved in the hydrolysis of the medium phytate and play an important role in the improvement of plant growth by the alleviation of the P stress when the phytate was used as the sole P source. This study could also have significant implication for improving crop production on low-P soils, which is a serious agronomic limitation worldwide.

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