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Differential Responses of Rice Acid Phosphatase Activities and Isoforms to Phosphorus Deprivation

Jeong Hyun Lim[†,§], Ill-Min Chung[‡,§], Sang Soo Ryu[†], Myoung Ryoul Park[†] and Song Joong Yun[†,*]

Faculty of Biological Resources Sciences and [†]Institute for Agricultural Science and Technology,
Chonbuk National University, Chonju 561-756, Korea

[‡]Research Team of Friendly Environmental Low-Input Natural Herbicide New Material Study, Konkuk University, Seoul 143-701, Korea

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Acid phosphatases (APases) play a role in the release of phosphate in organic complexes in soil. We investigated tissue- and isoform-specific responses of APases to phosphorus (P) deficiency in three rice genotypes; Dasan-byeo, Sobi-byeo, and Palawan. The levels of shoot APase activity per protein were similar in the three genotypes. They significantly decreased with P deprivation that was longer than seven days. Root APase activity per protein was two- to three-fold higher in Dasan than in Sobi and Palawan. In all genotypes the APase activity increased in P-deficient plants, but the increase was higher in Sobi and Palawan. After 21 days of P deprivation, secreted APase activity increased more than eight-fold in Dasan and two-fold in Sobi and Palawan. Isoform profiles of shoot and root APases were most diverse in Dasan. The activities of the major isoforms in P-deficient shoots decreased in all three genotypes. Depending on the genotypes, further increases in constitutive isoforms and new induction of one to four isoforms occurred in P-deficient roots. The results indicate that tissue and genotype differences in the response of APase to P deficiency are primarily facilitated by the different responses of the isoforms.

Keywords: Acid phosphatase, Phosphorous, Rice

Introduction

Phosphorus (P) is a major macronutrient required for plant growth and development. It plays not only an essential role in energy transfer and metabolic regulation, but it is also an important structural constituent of many molecules, such as nucleotides, phospholipids, and sugar phosphates. Inorganic phosphate (Pi) is the primary source of P in the soil, and it is the major form of P that is actively absorbed by plants. However, most of the P in soil is present as phosphate esters or metal ion salts which are not readily available to plants. The concentration and mobility of phosphate in the soil are lower than in other major nutrients (Clarkson and Lüttge, 1991), and P availability is often limited in native soils.

Under P-deficient conditions, plants show various morphological and biochemical adaptive responses. Phosphate uptake and the secretion of phosphatases and low-*Mr* organic acids increase in plants under low P conditions. The increased secretion of APases can help liberate phosphate in organic complexes (Duff *et al*., 1994; Marschner, 1995).

Phosphatases are often classified as acid or alkaline phosphatases, depending on whether their optimal pH for catalysis is below or above pH 7.0 (Vincent *et al*., 1992). Plant APases, generally with low substrate specificity, may be particularly important in the release of Pi from phosphate esters. Intracellular APases are primarily found in vacuoles. They are involved in the remobilization of P during developmental changes or P stress conditions (Duff *et al*., 1994). APases in the leaf have been used as biochemical markers for the genetic analysis of rice germplasm. They are grouped into four typical types, depending on their isoform patterns (Chern and Katayama, 1982). APases are also found in aleurone particles of rice grains, and their roles have been associated with the hydrolysis of phosphate reserves (Yamagata *et al*., 1979). However, only a few reports have investigated the response of APase activities to P stress in rice (Tadano and Saki, 1991; Ni *et al*., 1996). APase activity in roots and the secretion of APases from the roots increase in response to P deficiency (Tadano and Saki, 1991; Ni *et al*., 1996). There is, however, little information available on the tissue- and isoform-specific responses of APases to P deficiency. In this study, we investigated the tissue-

[§]The two authors contributed equally to this work

*To whom correspondence should be addressed.
Tel: 82-63-270-2508; Fax: 82-63-270-2640
E-mail: sjyun@moak.chonbuk.ac.kr

and isoform-specific responses of APases to P deficiency in three rice genotypes.

Materials and Methods

Chemicals and plant materials The chemicals used in this experiment were purchased from Sigma (St. Louis, USA). Three rice varieties [Dasan-byeo (Dasan, japonica x indica type), Sobi-byeo (Sobi, japonica type), and Palawan (indica type)] were used in this study and selected because they represented the three major cultivated rice biotypes. Seeds were germinated for three days in the dark at 25°C after 1 min surface sterilization in a 0.1% $HgCl_2$ solution (Yoshida *et al*., 1976). Uniform germinated seedlings were used for the hydroponic culture. The experiments were performed with three replicates. Paired t-tests were conducted for the normal and P deprivation treatments using statistical analysis software (SAS 6.12, Cary, USA).

Solution culture The composition and concentration of each element in the standard solution were as follows: N (3.6 mM), P (0.4 mM), K (1.3 mM), Ca (1.3 mM), Mg (2.0 mM), Fe (44.6 μM), B (25.0 μM), Mn (11.0 μM), Mo (0.7 μM), Zn (0.2 μM), Cu (0.2 μM), and citric acid (70 μM) (Yoshida *et al*., 1976). The seedlings were grown in an aerated solution in a glasshouse at 25°C/18°C (day/night) under a natural photoperiod (about 16 h) for three weeks. They were then transferred to the P-free nutrient solution and subjected to P deprivation for three weeks. The control plants were maintained for the entire period in the standard solution. The P-free nutrient solution was prepared similarly to the standard solution, except that NaH_2PO_4 was omitted. The solutions were changed every 3 to 4 d, and the pH adjusted daily to 5.0 with NaOH. The samples were taken 3, 7, 14, and 21 d after the treatment, ground in liquid nitrogen, and kept at −70°C until use.

APase assay Intracellular proteins were prepared using an extraction buffer containing 100 mM sodium acetate pH 6.8, 100 mM phenylmethylsulfonyl fluoride, 5 mM dithiothreitol, 10% glycerol, and 0.8% polyvinylpolypyrrolidone. The proteins that were secreted from the roots were obtained by incubating the seedlings in a plain medium containing 0.025% penicillin for 30 to 60 min at room temperature (Yun and Kaeppler, 2001). Intracellular and secreted protein solutions were used for APase and protein assays. Intracellular and secreted APase activities were determined using *p*-nitrophenol phosphate as the substrate (Yun and Kaeppler, 2001). The assay mixture contained 100 mM sodium acetate, pH 4.5, 40 mM *p*-nitrophenol phosphate, and 25 μg of the sample protein. The mixture was incubated at 37°C for 10 min. The reaction was terminated by adding NaOH to a final concentration of 1.5 M. The absorbance of the solution was measured at 410 nm. The APase isoforms were separated on 10% non-denaturing polyacrylamide gels (Baek *et al*., 2000) and stained with a mixture consisting of 50 mM sodium acetate, pH 5.5, 1 mg/ml Fast Black K salt, 10 mM $MgCl_2$, and 0.03% (w/v) beta-naphtyl acid phosphate overnight at room temperature (Aarts *et al*., 1991). The protein contents in the extract were determined using the method of Bradford (1976) with bovine serum albumin as the standard.

Results

Shoot APase activity The rice seedlings grew normally in a standard nutrient solution. The ones that were subjected to P deprivation for three weeks showed few visible symptoms of P deficiency, but the percentage of fresh weight (FW) that was allocated to the roots increased in plants after 7 d of P deprivation in all genotypes. The levels of shoot APase activity per FW were higher in Dasan and Palawan than in Sobi. Also, activities decreased over the treatment period in all three genotypes. The effect of P deficiency on the shoot APase activity varied within a relatively narrow range and depended on the treatment period and genotype. The relative shoot APase activity in the P-deprived plants when compared to the P-sufficient plants (P-/P+ ratio) varied between 0.8 and 1.1 (Fig. 1A). The APase activity per protein, from 7 d of P deprivation onwards, was always less in the P-deficient shoots than in the P-sufficient shoots; therefore, the P-/P+ ratio of activity ranged from 0.68 to 0.95 (Fig. 1B).

Root APase activity Large differences in root APase activity were observed among the three genotypes, with activity per FW of Dasan being two- to three-fold higher than for Sobi and Palawan. The APase activity in the roots was more than two-fold higher than in the shoots of Dasan, but similar in both tissues in Sobi and Palawan. The relative APase activity per FW increased under P deprivation for more than 14 d, and was higher for Sobi than Dasan and Palawan (Fig. 2A). The levels of APase activity per unit protein in the P-sufficient plants increased significantly in Sobi and Palawan, and decreased after 21 d for all genotypes. The APase activity increased in the P-deprived roots of all genotypes, but the rate was higher in Sobi and Palawan. This resulted in relatively higher P-/P+ activity ratios than for Dasan (Fig. 2B).

Secreted APase activity The effect of P deficiency on secreted APase activity in roots per FW varied with time and genotype. In Dasan, however, the P-/P+ ratio of activity constantly increased, up to 2.6 under P deficiency, even though the absolute level of APase activity decreased in both the P-deficient and P-sufficient plants after 14 d of treatment (Fig. 3A). The most drastic change in APase activity under P deficiency was observed in secreted APase per unit protein. The APase activity in P-deficient plants increased until 14 d of treatment in the three genotypes. It increased more than eight-fold in Dasan and more than two-fold in Sobi and Palawan after 21 d of P deprivation (Fig. 3B).

APase isoforms Changes in the activity and profile of APase isoforms appeared 3 d after P deprivation (data not shown), and became apparent 21 d after the treatment. Isoform profiles of the shoot and root APases were more variable in Dasan. In shoots and roots, the three fast-moving major isoforms (isoforms 1, 2, and 3) were commonly found

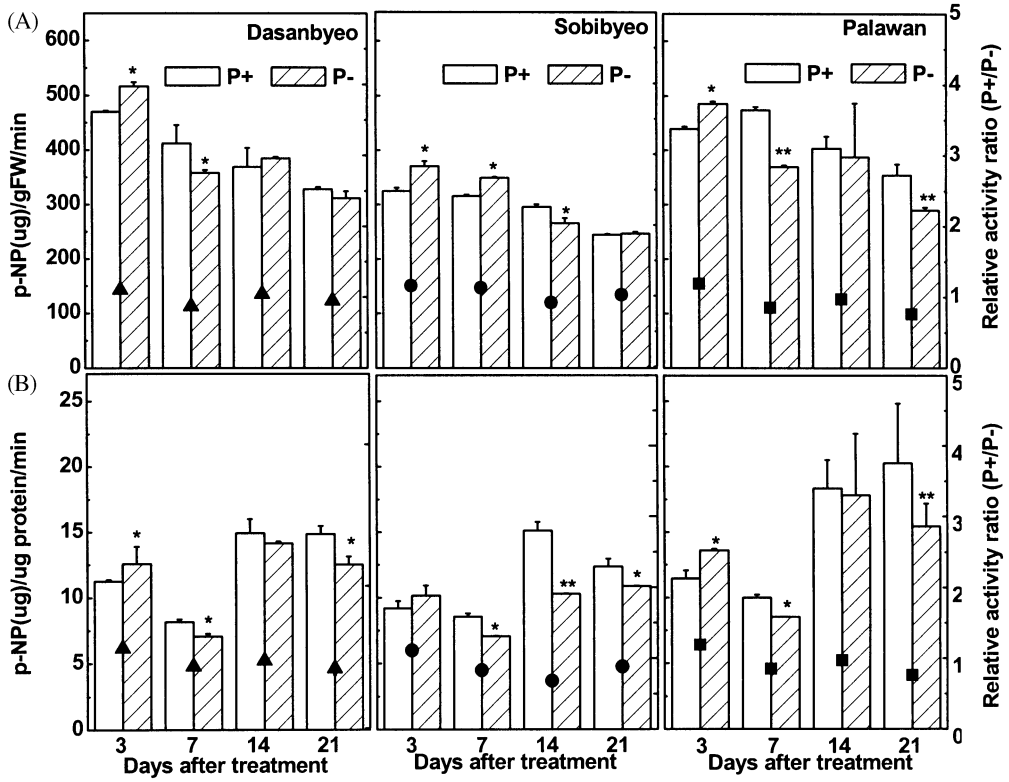

Fig. 1. The response of intracellular acid phosphatase in shoots to phosphorus deprivation. Enzyme activity was expressed based on unit fresh weight (A) and unit protein basis (B). The relative values of shoot APase activity in P-deprived plants when compared to P-sufficient plants (P-/P+ ratio) for Dasan (▲), Sobi (●), and Palawan (■) are as indicated. Three-wk-old seedlings were grown additional days as indicated in the standard (P+) or P-free (P-) solutions. * and **, the paired means of the P+ and P- treatments were significantly different at $P<0.05$ and $P<0.01$, respectively. P-NP, p-nitrophenol blue; D, Dasan-byeo; S, Sobi-byeo; P, Palawan.

in the three genotypes (Fig. 4). In Dasan, one major isoform of APase (isoform 9) was detected in the shoots, while two slow-migrating major isoforms (isoforms 9 and 10) were detected in the roots. The activity of the major isoforms in P-deprived shoots decreased in the three genotypes. Isoform 10 was slightly induced in P-deprived shoots, but only in Dasan. In roots, the minor isoform 7 was induced under P deficiency in the three genotypes. Other minor isoforms (isoforms 9, 10, and 11 in Sobi, and isoforms 6 and 8 in Palawan) were also induced. The activity of the two major slow-moving constitutive isoforms (isoforms 9 and 10) was very high and increased even further. However, isoform 1 decreased in P-deprived roots of Dasan (Fig. 4).

Discussion

Reduced growth and yield are the overall agronomic responses of rice plants to P deficiency. However, this reduced growth and yield is determined by the many physiological and biochemical changes in plants under P deficiency. The response of APase activity to short-term P deprivation differs between the shoots and roots. There was decreased intracellular APase activity per protein in shoots as a result of P deprivation for more than 7 d, after a transient small increase 3 d after the treatment began (Fig. 1). This decrease in the level of shoot APase activity was the result of the induction of the major isoforms at lower levels in the P-deprived leaves (Fig. 4). The decrease in APase activity from 7 d after P deprivation is early when compared to the responses of APases in other plant species. For example, APase activity increases in the leaves of Arabidopsis (Trull *et al*., 1997), the common bean (Yan *et al*., 2001), and maize (Yun and Kaeppler, 2001) at least 14 d after P deficiency. In tomato cells, however, the level of intracellular APase activity does not increase as a result of P deficiency (Goldstein *et al*., 1988).

Intracellular APase activity per unit protein increased in rice roots under P deprivation for 21 d (Fig. 2). This increase was contributed to by an increase in the constitutive isoforms as well as the induction of new isoforms under P deprivation (Fig. 4). Ni *et al*. (1996) reported increased APase activity in rice roots under P deficiency, but there was no information on the responses of isoforms in other tissues. Similar increases in APase activity in P-deficient roots have been observed in Arabidopsis (Trull *et al*., 1997) and maize (Yun and Kaeppler, 2001).

Secreted APases have been extensively investigated in

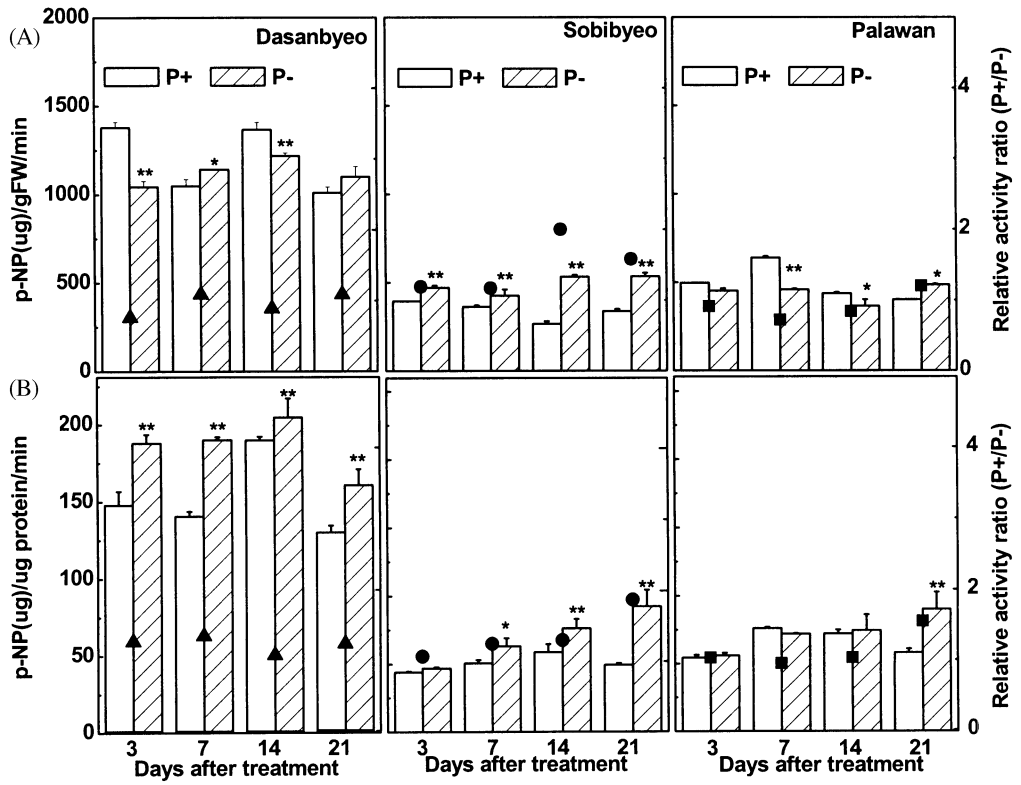

Fig. 2. The response of intracellular acid phosphatase in roots to phosphorus deprivation. Enzyme activity was expressed based on unit fresh weight (A) and unit protein (B). The relative values of root APase activity in P-deprived plants when compared to P-sufficient plants (P-/P+ ratio) for Dasan (▲), Sobi (●), and Palawan (■) are as indicated. Three-week-old seedlings were grown additional days as indicated in the standard (P+) or P-free (P-) solutions. * and **, the paired means of the P+ and P- treatments were significantly different at $P<0.05$ and $P<0.01$, respectively. P-NP, p-nitrophenol blue; D, Dasan-byeo; S, Sobi-byeo; P, Palawan.

relation to P deficiency in many plant species. Secreted APase activity per unit protein also increased in roots under P deprivation for 14 to 21 d. Although the secretion of APase under P deficiency is common in many plant species, including rice, the level of secretion differs greatly among species (Tadano and Sakai, 1991). The lupin and tomato are known to secret exceptionally high levels of APases (Tadano and Sakai, 1991; Watt and Evans, 1999; Miller *et al.*, 2001), while monocots (such as rice, wheat, and maize) secrete a relatively low level of APases (Tadano and Sakai, 1991; Yun and Kaeppler, 2001).

There were genotypic differences in the responses of APases to P deficiency in rice. APase activities in shoots and roots were similar in Sobi and Palawan, but about three-fold higher in the roots than the shoots of Dasan. Intracellular root APase and secreted APase activity per protein were significantly higher in Dasan than in Sobi and Palawan. High levels of APase activity in Dasan were contributed to by the two prominent major isoforms, whose activities increased further under P deprivation (Fig. 4). This suggests that genotypic differences in the level of APase activity are facilitated by differences in the profiles and activities of isoforms.

Genotypic differences in the responsiveness to P deficiency are more drastic in the secreted APase. Even though there is a stage-specific variation, it is apparent that the secretion levels of APase are only slightly affected by the constitutive levels of secreted APase. This is because the secreted APase activities in P-sufficient plants were similar across the genotypes. Isoforms secreted from rice roots are unknown. Since the secretion of APase occurs in P-sufficient plants as well as in P-deficient plants, then it is likely that one or more of the constitutive and induced isoforms are secreted. Further investigations on the secreted APases will enhance our understanding of their secretion processes.

Numerous reports document the induction and secretion of APase in P-stressed plants and the significance of the changes. Intracellular APases may have a role in phosphate recycling (Smyth and Chevalier, 1984; Duff *et al.*, 1994). Secreted APases play a role in the hydrolysis of Pi from organic P compounds in soil (Goldstein *et al.*, 1989; Lefebvre *et al.*, 1990). Therefore, increased intracellular and secreted APases can contribute to enhancing P uptake and utilization under P-limited conditions. However, no significant relation between the level of leaf APase and adaptation to low P availability was observed in the common bean (Yan *et al.*, 2001). P use

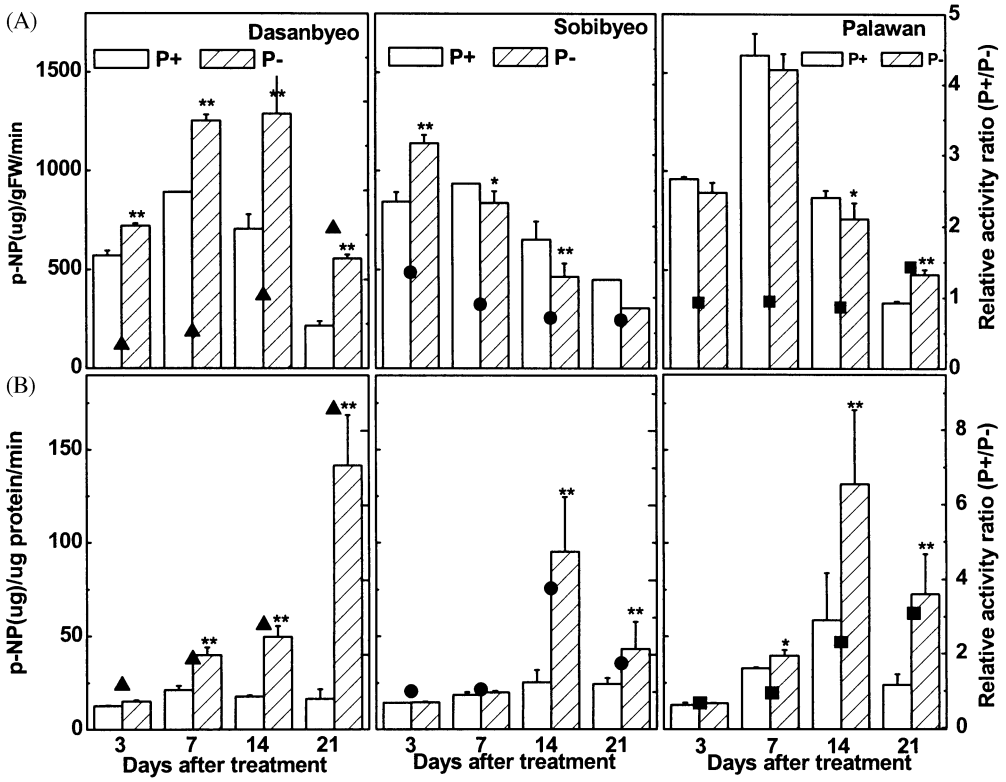

Fig. 3. The response of secretory acid phosphatase activity to phosphorus deprivation in roots. Enzyme activity was expressed based on root fresh weight (A) and total secreted protein (B). The relative values of root APase activity in P-deprived plants when compared to P-sufficient plants (P-/P+ ratio) for Dasan (▲), Sobi (●), and Palawan (■) are as indicated. Three-week-old seedlings were grown additional days as indicated in the standard (P+) or P-free (P-) solutions. * and **, the paired means of the P+ and P- treatments were significantly different at $P<0.05$ and $P<0.01$, respectively. P-NP, p-nitrophenol blue; D, Dasan-byeo; S, Sobi-byeo; P, Palawan.

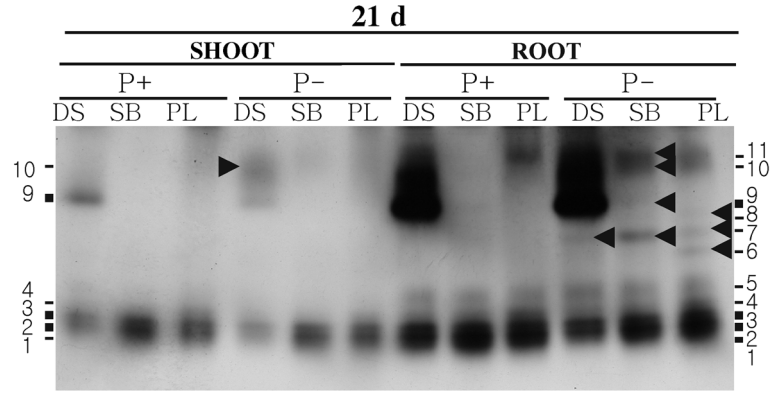

Fig. 4. The response of acid phosphatase isoforms in the shoots and roots to phosphorus deprivation. Twenty micrograms of protein from seedlings grown for 3 wk in the standard (P+) or P-free (P-) solutions were separated on a 10% polyacrylamide gel, and the acid phosphatase activity was stained. Arrowheads indicate isoforms that are induced under P deprivation. DS, Dasan-byeo; SB, Sobi-byeo; PL, Palawan.

efficiency is a complex trait that is associated with coordinated networks of acquisition, utilization, and remobilization processes. Nevertheless, it is highly likely that increased secreted APases can contribute to P acquisition since the liberation of Pi from organic complexes (Duff *et al*., 1994; Marschner, 1995) is an essential step for P uptake in plants.

The response of APase to P deficiency can be expressed as the P-/P+ ratio of APase activity. The P-/P+ ratio of intracellular APase activity of shoots was similar in the three genotypes, but significantly higher in the roots of Sobi and Palawan. In Sobi and Palawan, the induction of new isoforms seems to be a major adaptive response to P deficiency.

Although the response of APases to P deprivation differed among the genotypes, the absolute APase activity that increased under P deficiency was similar in the three genotypes. Consequently, the final APase activity levels were significantly affected by the constitutive levels of APases in tissues and genotypes. Further investigation on the relationship between APase activity and P availability from organic phosphate may provide additional information on both the role of APase and efficiency of genotypes in P acquisition.

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