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# BIOCHEMICAL AND MOLECULAR ADAPTATION OF RICE PLANTS TOWARDS LOW PHOSPHATE CONDITION

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## ABSTRACT

Rice is the staple food for half of the world's population. Phosphorus is an essential element for rice plant growth, development and production; however, the availability of soluble phosphate for plant uptake is often limited due to the binding with cation in different pH environments. Therefore, the development of rice plant varieties, which can grow well under a limited phosphate environment, is an urgent need for agricultural development for biotechnology. This study reported two contrasting rice varieties' biochemical and molecular adaptation toward a low phosphate medium. The results show that the response of these two varieties is genotype-dependent. We observed a higher phosphate use efficiency in the low-Pi-sensitive G299 rice variety than in the low-Pi-tolerant G22 rice variety.

Moreover, the expression of five out of six investigated genes relating to the low Pi responsive pathway, including two purple acid phosphatase (*PAP),* phosphate starvation response 2 (*PHR2),* *SPX* (*SYG1/PHO81/XPR1*) domain-containing gene, phosphate transcription factor 1 (*PTF1)* and *phosphate 1* (*PHO1)* were significantly higher in the low-Pi-sensitive accession than in the tolerant genotype. Impressively, 214 times more expression of the *PHO1* was observed in the leaves of the G299 variety than in the G22 variety, followed by *SPX*, *PTF1*, *PHR2,* and *PAP21* genes. Similarly, the *PHO1* was induced at the highest level in the roots of the G299 variety, followed by *PAP21*, *PHR2*, *PTF1*, and *SPX1* genes. These findings supply further information for elucidation of the mechanism behind the response of rice plants to low phosphate medium and the development of the low Pi nutrient happy rice varieties.

**Keywords:** Low-phosphate-responsive genes, low-Pi sensitive variety, low-Pi tolerant variety, *Oryza sativa*, phosphate starvation, phosphate use efficiency.

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## INTRODUCTION

Rice is a staple food for half of the world's population. The increased speedy world population, estimated to reach more than 9 billion people by 2050, leads to an urgent need to increase rice production twice compared to now to meet the requirement (Hunter et al., 2017). Besides the biotic stress that severely affects rice productivity, the abiotic stress also contributes to rice production loss. Phosphate (Pi) plays a central role in rice growth, development and reproduction. Without or with a deficient Pi supply, rice plants will have severe problems in plant growth, the number of panicles, and productivity (Balemi & Negisho, 2012). Pi is the main component of the nucleic acid or phosphor-lipid layer in the cell membrane and involves the essential processes in the cell, including photosynthesis and ATP synthesis (White & Hammond, 2008). However, the statistic shows that more than 70% of cultivated land is Pi deficiency (Kirkby & Johnston, 2008). There are several reasons for Pi starvation. First is the abuse used by farmers worldwide for the cultivated crop. Second is the problem of Pi fixing with aluminium and iron or calcium in the acidic or basic medium, which leads to the unavailable dissolve Pi for plant uptake even if the Pi source is still available. Consequently, only approximately 20–30% of the Pi applied is utilized by plants (Cordell et al., 2011). To cope with a low Pi medium, plants have developed several strategies from morphological to molecular adaptations, leading to changes in gene expression and biochemical levels. In terms of biochemical adaptation to low Pi, an increase in Pi use efficiency (PUE) is a common strategy of plants. The attention to PUE of plants has been raised by some scientists (Cordell et al., 2009; Cao et al., 2009), and securing PUE will be critical for future food security by a human.

In terms of molecular level, a number of genes involved in low Pi response have been identified in rice. The secretion of purple acid phosphatases or activation of Pi transporter genes is a typical first response (Abel et al., 2002). The purple acid phosphatases involve the solubilization of un-soluble Pi fixed to metals. Therefore, it will increase the availability of plant uptake (Liu, 2021). The purple acid phosphatase (*PAP)* genes are directly regulated by the transcription factor, namely phosphate starvation response 2 (*PHR2)* (Zhang et al., 2011). Therefore, *PHR2* is also considered one of the earliest effector responses to low Pi signals (Bustos et al., 2010). Another high-affinity phosphate transcription factor named *OsPTF1* is also involved in the Pi starvation response by acquiring Pi from the rhizosphere (Yi et al., 2005).

Moreover, the transporter to transfer Pi from the root to shoots during Pi starvation plays a significant role in maintaining Pi homeostasis. *OsPHO1*, a homolog of *AtPHO1* in *Arabidopsis* and belongs to the *phosphate 1 (PHO1)* gene family, transfers Pi from the root to shoots (Secco et al., 2010). Furthermore, 18 genes relating to PUE, including five Pi transporters, two auxin response factors, three SYG1/Pho81/XPR1 (SPX) domain-containing proteins, and two MYB-like transcription factors (TFs), and a phosphatase, also have been reported (Yamamoto et al., 2012). In 2012, an important discovery of the *phosphorus starvation tolerance* 1 *(Pstol1)* gene in a low-Pi-tolerant rice variety was announced, in which the overexpression of this gene in Nipponbare and IR64 reference rice accession led to the increase of 60% productivity of transgenic plants (Gamuyao et al., 2012). However, several studies after showed that this *Pstol1* gene increases rice productivity, but it was not the only way. There were alternative genes (Vigueira et al., 2016; To et al., 2020).

To secure global food, the urgent need is to develop the low Pi nutrient happy rice varieties, which can be supported by research on the molecular and biochemical basis of Pi use. Therefore, in this study, two selected rice varieties, which morphologically responded contractedly to low Pi (Mai et al., 2021), were

used as plant materials to investigate the phosphate used efficiency of these rice varieties. Then, the molecular mechanism underlies the efficiency of Pi used was investigated.

## MATERIALS AND METHODS

### Plant materials

Two Vietnamese rice varieties, namely G22 and G299, were used as plant materials. Their seeds were donated by the Plant Resources Center in Hanoi, Vietnam.

### Plant cultivation

The conditions for plant culture followed the description by To et al. (2020). Briefly, the seeds of the two selected Vietnamese rice varieties were first incubated in an oven at 45 $^{o}$C for 5 days to break down seed dormancy. Then, they were germinated at 37 $^{o}$C over 7 days in high humidity container. The plantlets with similar heights were selected to grow in the culture room at 28 $^{o}$C and approximately 70–80% humidity. Rice plants were grown in Yoshida hydroponic culture medium in two conditions: full phosphate (P0) at 320 µM P and phosphate starvation (P*) at 10 µM P. The plants were irrigated with either a full Pi medium for the control experiment or a low Pi medium for the treatment experiment. The supplementation of media was implemented every week for six weeks.

### Phosphate use efficiency quantification

After growing in a hydroponic culture medium for six weeks in two cultural conditions, the plants were harvested and dried in an oven at 70 $^{o}$C for one week to reach the constant weight. Then, the leaves of plants were weighted to have shoot weight (SHW) and ground into a fine powder using a tissue lyser machine. The quantification of Pi in shoots of plants grown under full and low Pi was followed protocol by To et al. (2020). $KH_2PO_4$ and vanadate–molybdate reagents were first used to build the standard curve with different Pi concentrations. Then, 0.3 g of dry leaf sample was ashed for 6 h in a Muffle furnace (Nabertherm, New Castle, DE, USA). The reagent vanadate–molybdate comprised ammonium molybdate, ammonium metavanadate and concentrated hydrochloric acid (Xilong Chemical Co., Ltd.; 36.0−38.0%). The principle of this method lies under vanadate–molybdate reagent and the dissolved sample, which contains the released form – orthophosphate ready to react with ammonium molybdate under hydrochloric acid conditions to form a molybdophosphoric acid. In the presence of vanadium, yellow vanadomolybdophosphoric acid is formed. Hence, the yellow colour's intensity is proportional to the phosphate concentration present in the leaf sample. The absorbance of the assay solution was read at a wavelength of 420 nm by a UV-1800 UV-VIS spectrophotometer (Shimadzu). The Pi concentration in the samples was determined based on the optical density values obtained and the standard curve with its linear regression line.

The concentration of Pi in shoot samples (mg) was calculated by multiplying the Pi concentration (mg/g) in each sample by SHW (g).

The PUE (g SHW/mg Pi) was calculated by dividing the SHW by Pi concentration at given Pi conditions (Hammond et al., 2009).

$$\text{PUEhigh Pi} = \frac{\text{SHWhigh Pi}}{[\text{Pi}]\text{high Pi}}$$

$$\text{PUElow Pi} = \frac{\text{SHWlow Pi}}{[\text{Pi}]\text{low Pi}}$$

### RNA extraction, cDNA synthesis, and real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis

At six weeks, the leaf and root samples of G22 and G299 cultivars grown in the Yoshida hydroponic culture with full and low Pi treatment were collected. TRIzol reagent (Thermo Scientific, Waltham, MA, USA) was used to extract total mRNA from samples. DNAse I (Thermo Scientific) was used to remove all DNA from the extracted mRNA before cDNA synthesis using the MaximaR First Strand cDNA Synthesis Kit

(Thermo Scientific). Analytik Jena qPCR system was used to quantify and compare the expression of some interesting genes in the two selected rice varieties grown under two different cultural media. The experiment was performed in triplicate per sample per time point per treatment using the Go Taq RT-qPCR master mix (Promega, Madison, WI, USA). qPCR was performed for six genes related to the Pi pathways. The primers were designed using the website https://www.ncbi.nlm.nih.gov/tools/primer-blast then synthesized and displayed in Table 1. Relative gene expression levels were normalized to the reference gene (actin). In the 15 µL reaction mix, there were 4 µL 2× master mix (Promega), 250 nM forward primer, 250 nM reverse primer, 400 ng DNA template, and $H_2O$ to make up the volume to 15 µL. The thermal cycling conditions were as follows: 95 $^oC$ for 5 min, 40 cycles at 95 $^oC$ for 30 s, 56 $^oC$ to 58 $^oC$ for 1 min, and 72 $^oC$ for 1 min. The $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001) was used to calculate the relative gene expression under different conditions.

*Table 1.* List of primers was used to amplify the genes related to Pi responses by qRT-PCR

| No. | Name | Forward primer | Reverse primer |
|---|---|---|---|
| 1 | *SPX1* | CGGTTTCTGTTGGCAGTTGG | CATACGCTGCCCTGCTTACT |
| 2 | *PHR2* | AAGCACACCTATGCCACCTC | CAACAGACCCGGTACTGGAC |
| 3 | *PAP21* | AAGAGGAATCGAGGACAAGA TATTTG | AAGCCTCTTCTTGTTCGGATCA |
| 4 | *Pstol1* | ATGCTGCTCTGTCAAAGGGCAT | CAAGCTCAAAGCCCTTTTGGTG |
| 5 | *PTF1* | TGTTGACGGTTCGTCGGAAT | GCAAGCATAGTGGGGTTCCT |
| 6 | *PHO1.2* | ACTGGATTTCTCGCTCGCTT | GCAATCCCATAAACCATTTCACCA |
| 7 | *Actin* | CAACACCCCTGCTATGTACG | CATCACCAGAGTCCAACACAA |

**Statistical analysis**

The differences between investigated parameters were statistically analyzed using the Student's *t*-test in R program software version 3.6.

## RESULTS

**Phosphate use efficiency of rice plants grown under the low Pi condition**

Two contrasting rice varieties that responded to low Pi in terms of morphological traits were chosen to investigate how the PUE of these two varieties were adapted to the Pi starvation medium. After six weeks grown in two different media in terms of Pi concentration, the results showed that under full Pi medium, there was no significant difference in PUE of the two rice varieties ($p > 0.05$). However, significant differences between G22 and G299 varieties in PUE were found when rice plants were grown under a low Pi medium ($P < 0.001$). The PUE was higher in the low-Pi-sensitive G299 variety than in the low-Pi-tolerant G22 variety (Fig. 1).

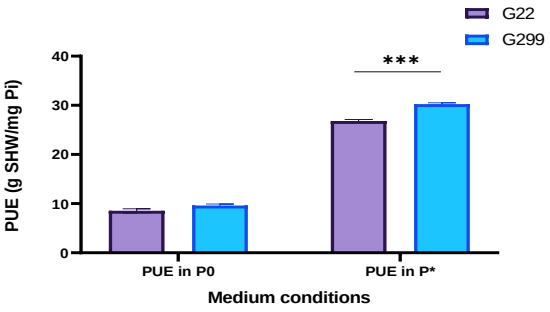


*Figure* 1. Phosphate use efficiency (PUE) in two Vietnamese rice varieties under full (P0) and low phosphate (P*) conditions. Values were presented by mean ± standard deviation from three independent biological replicates. (***) indicates a significant difference of $p < 0.001$ according to Student's *t*-test. G22 (Trung Trang Tuyen Quang accession belongs to *Indica* group, VNPRC code is 760), G299 (Blao Sinh Sai accession belongs to *Japonica* group, VNPRC code is 4806)

**Relative expression of Pi-related genes in G22 and G299 rice accessions under low and full Pi conditions**

In all five investigated genes, excepting *Pstol1*, in both leaves and roots, the expression level of the genes was detected much higher in low-Pi sensitive G299 rice variety than in low-Pi tolerant G22 one (Figs. 2, 3). Impressively, approximately 214 times more expression of *PHO1* was observed in the leaves of the G299 variety than in the G22 variety (Fig. 2). Moreover, under low Pi conditions, the expression of the *SPX1* gene was also induced stronger in the leaves of G299 than in G22. The low Pi condition caused approximately 18 times more the expression of the *SPX1* gene in leaves of the G299 variety than in the G22 variety (Fig. 2). The expression level of *PTF1*, *PHR2*, and *PAP21* genes were observed at 10, 16,3 and 33.3 times higher in the leaves of the G299 variety than in the G22 variety, respectively. However, no significant difference in the expression of the *Pstol1* gene was observed in leaves of the G299 variety compared to the G22 variety ($p > 0.05$) (Fig. 2).

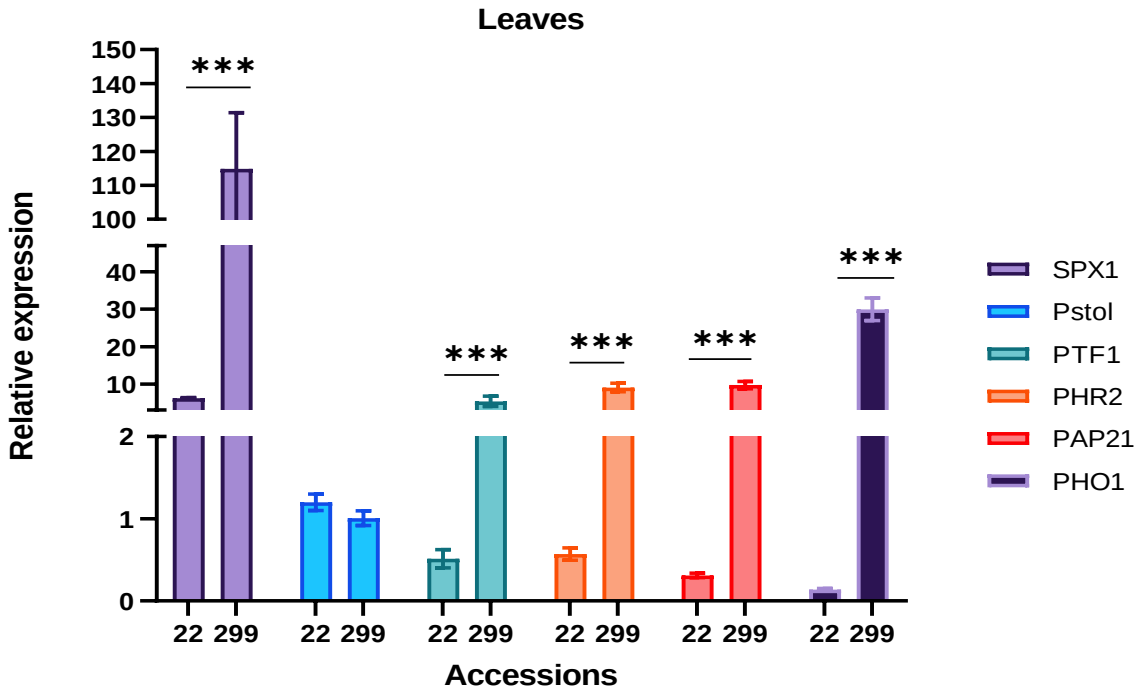


*Figure 2*. Relative expression of Pi-related genes in leaves of 6-week-old rice plants grown under low Pi conditions (10 µM P). The expression was normalized with the expression of the housekeeping gene *Actin*. Values were presented by mean ± standard deviation from three independent biological replicates. (***) indicates a significant difference $p < 0.001$ according to Student's *t*-test

A similar situation was observed in the roots of the G299 variety compared to the G22 variety. A significantly higher expression of five out of six investigated genes was observed in the roots of the G299 variety compared to the G22 variety ($p < 0.001$) (Fig. 3). Remarkably, approximately 24.3 times more in the expression of gene *PHO1* was detected in the roots of G299 than G22 variety. The expressions of *PAP21*, *PHR2*, *PTF1*, and *SPX1* were 10.2, 4.9, 4.45, and 4 times significantly higher in G299 than G22 variety, respectively ($p < 0.001$) (Fig. 3).

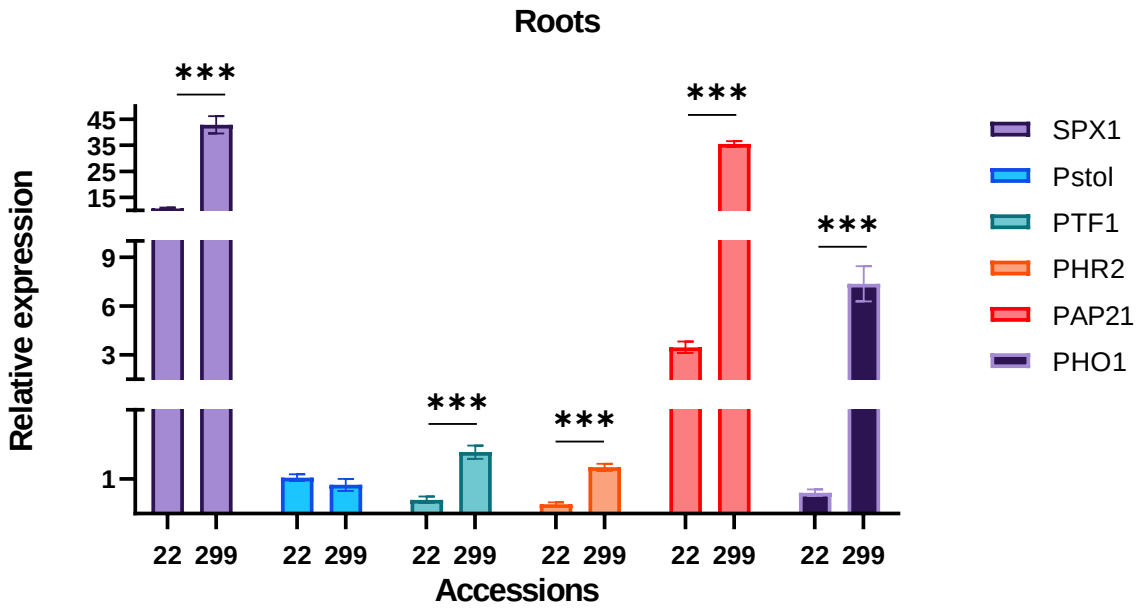


*Figure 3*. Relative expression of Pi-related genes in roots of 6-week-old rice plants grown under low Pi conditions (10 µM P). The expression was normalized with the expression of the housekeeping gene Actin. Values were presented by mean ± standard deviation from three independent biological replicates. (***) indicates a significant difference $p < 0.001$ according to Student's *t*-test

## DISCUSSION

This study is the first investigation of the phosphate utilization efficiency of two contrasted Vietnamese rice varieties (G22 and G299), which responded morphologically differently towards low Pi medium, and was managed under low Pi medium. Then, the expression of Pi-related genes in these two rice varieties was also studied to understand the response not only morphological but also the biochemical and molecular levels of these two rice varieties toward low Pi conditions.

The results showed that PUE was significantly higher in the low-Pi-sensitive rice G299 variety than in the low-Pi-tolerant G22 variety in low Pi conditions. These results imply that the low-Pi-sensitive G299 variety always activated its pungent mechanisms than the low-Pi-tolerant G22 variety to uptake and use Pi more efficiently under low Pi conditions.

Regarding Pi-related gene expression, in leaf and root samples, five out of six investigated genes, including *SPX1*, *PTF1*, *PHR2*, *PAP21* and *PHO1*, showed significantly higher expressions in the low-Pi-sensitive variety than the low-Pi-tolerant variety ($p < 0.001$).

The *SPX1* gene was induced strongest in leaves, followed by the *PHO1* gene, showing that these two genes were strongly involved in the responsive pathway toward a low Pi condition in rice. The expression of the *SPX1* gene was high in the roots; however, the level was lower than in the leaves. In other studies, *SPX1*, *SPX2* (Wang et al., 2009), *SPX4* (Qundan et al., 2014), and *SPX6* (Zhong et al., 2018) were also induced their expressions when rice plants were grown in a low Pi medium. In *Arabidopsis*, *PHO1* played a crucial role in Pi efflux, and its expression was strongly induced under low Pi conditions (Stefanovic et al., 2007, 2011). In rice, *PHO1* also plays an essential role in Pi distribution (Secco et al., 2010).

Furthermore, the secretion of purple acid phosphatases was considered another strategy

to deal with Pi starvation in plants. In our study, higher expression of *PAP21* was recorded in low-Pi-sensitive variety, and it was higher in roots than in leaves. In tomatoes under long-term Pi starvation, acid phosphatases were secreted from roots into the culture medium to degrade extracellular organic P compounds, unabsorbable for plants (Bozzo et al., 2006). In rice, many *PAP* genes were significantly induced under Pi starvation conditions (Zhang et al., 2011). Recently, Mehra et al. (2017) also indicated that *OsPAP21b* primarily responds to Pi deficiency by increasing its expression under this medium (Mehra et al., 2017).

*PHR2* was slightly higher induced in roots samples of G299 than G22 rice accession, but in root samples, it was induced approximately 20 times in G299 compared to G22. Similarly, the *PTF1* gene was induced slightly in roots, but it increased the expressions detectably in leaves of the G299 compared to the G22 rice variety. *PTF1* was also induced in roots when rice plants were grown in a low Pi medium. The transgenic rice plants that overexpressed the *PTF1* gene enhanced the tolerance to Pi starvation and increased 30% of Pi content in rice plants (Yi et al., 2005). Similarly, the overexpression of *PHR2* in rice increased the accumulation of Pi in shoots under Pi-insufficient conditions, showing the critical role of *PHR2* in plants in response to low Pi conditions. Reduction of expression of *PHR2* results in lower expression of several low-Pi-response genes under low Pi conditions (Zhou et al., 2008).

Overall, the study showed that the response of rice plants to low Pi is genotype-dependent. We observed the strong response of rice plants to low Pi medium in the low-Pi sensitive G299 variety, but smaller changes in terms of chemical and molecular levels were recorded in the low-Pi-sensitive G22 variety. This observation was also observed in other studies (He et al., 2003). This phenomenon can be explained by the differences in living conditions of *indica* and *japonica* subspecies, which led to the development of a number of different strategies, including physiological, biochemical and molecular characteristics, to deal with low Pi conditions (Yang et al., 2014; Liu, 2021).

This study is the first study that shows how the PUE of two Vietnamese contrasted rice varieties were monitored under low Pi conditions. Then, the expression of some essential genes involved in the low-Pi-responsive pathway was also investigated, which opens up exciting avenues for future research.

## CONCLUSION

The current study identified higher PUE in the low-Pi-sensitive G299 variety than in the low-Pi-tolerant G22 variety in low Pi conditions. The expression of five out of six investigated genes relating to the Pi pathway was significantly higher in the low-Pi-sensitive accession than in the tolerant genotype. These results are regarded as adaptations of rice plants to low Pi conditions to meet their needs. This study provided more knowledge on PUE and Pi-related gene expression of two Vietnamese contrasted Pi-efficient rice genotypes to respond to low Pi conditions. For the following steps, more studies should be implemented to find the essential genes responsible for the adaptation of rice plants to low Pi conditions to create the elite rice variety.

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