**Title: Phosphorus toxicity disrupts Rubisco activation and reactive oxygen species defence systems by phytic acid accumulation in leaves**

**Running title:** Mechanisms of phosphorus toxicity

**Authors: Daisuke Takagi1\*, Atsuko Miyagi2, Youshi Tazoe1, Mao Suganami1, Maki Kawai-Yamada2, Akihiro Ueda3, Yuji Suzuki4, Ko Noguchi5, Naoki Hirotsu6, Amane Makino1**

1Graduate School of Agricultural Science, Tohoku University, Aoba-ku, Sendai 980-8572, Japan

2Graduate School of Science and Engineering, Saitama University, Shimo-Okubo, Sakura-ku, Saitama 338-8570, Japan

3Graduate School of Integrated Sciences for Life, Hiroshima University, Hiroshima 739-8528, Japan

4Faculty of Agriculture, Iwate University, Morioka, Iwate 020-8550, Japan

5School of Life Sciences, Tokyo University of Pharmacy and Life Sciences, Horinouchi, Hachioji, Tokyo 192-0392 Japan

6Faculty of Life Sciences, Toyo University, 1-1-1 Izumino, Itakura-machi, Oura-gun, Gunma 374-0193, Japan

**\*For correspondence**

**Corresponding author:** Daisuke Takagi

E-mail: [daisuke.takagi.b4@tohoku.ac.jp](mailto:daisuke.takagi.b4@tohoku.ac.jp)

Tel: +81-22-757-4287/Fax: +81-22-757-4289

**Funding:** This work was supported by the Japan Society for the Promotion of Science (JSPS) research fellowship [JSPS KAKENHI Grant No. 18J00852 (DT) and KAKENHI Grant No.16H06379 (AMa)].

**ABSTRACT　(188 words)**

Phosphorus (P) is an essential mineral nutrient for plants. Nevertheless, excessive P accumulation in leaf mesophyll cells causes necrotic symptoms in land plants; this phenomenon is termed P toxicity. However, the detailed mechanisms underlying P toxicity in plants have not yet been elucidated. This study aimed to investigate the molecular mechanism of P toxicity in rice. We found that under excessive inorganic P (Pi) application, Rubisco activation decreased and photosynthesis was inhibited, leading to lipid peroxidation. Although the defence systems against reactive oxygen species (ROS) accumulation were activated under excessive Pi application conditions, the Cu/Zn-type superoxide dismutase activities were inhibited. A metabolic analysis revealed that excessive Pi application led to an increase in the cytosolic sugar phosphate concentration and the activation of phytic acid synthesis. These conditions induced mRNA expression of genes that are activated under metal-deficient conditions, although metals did accumulate. These results suggest that P toxicity is triggered by the attenuation of both photosynthesis and metal availability within cells mediated by phytic acid accumulation. Here, we discuss the whole phenomenon of P toxicity, beginning from the accumulation of Pi within cells to death in land plants.

**Key words:**

Land plants; phosphorus; reactive oxygen species; phytic acid; photosynthesis; plant nutrition

**Summary statements: (268 characters including spaces)**

Excessive phosphorus application inhibits photosynthesis and attenuates reactive oxygen species defence systems by stimulating phytic acid synthesis. These physiological responses initiate cell death by oxidative stress, which has been observed as phosphorus toxicity.

**Acknowledgements:**

The authors thank Editage (Cactus Communications Inc., <https://www.editage.jp/>) for English language editing of the present manuscript. The authors also thank Emer. Prof. Tadahiko Mae, Dr. Hiroyuki Ishida, and Dr. Keiki Ishiyama of Tohoku University for fruitful discussions on this manuscript. The authors thank Ms. Louise Thiaville of Tohoku University for her kind language support. The authors thank Prof. Hirohumi Saneoka of Hiroshima University for his technical support.

**Conflicts of interest:**

The authors declare no conflicts of interest.

**Author contributions:**

Conceptualisation: DT; investigation: DT, AMi, YT, MS, AU, YS, KN, MK, and NH; original draft: DT; writing, review, and editing: DT, AMi, YT, MS, AU, MK, YS, KN, NH, and AMa; funding acquisition: DT and AMa.

**INTRODUCTION**

In land plants, phosphorus (P) is one of the most essential macronutrients required to maintain their growth and reproduce seeds for the next generation. P is required in plant cells as a structural constituent of DNA, RNA, phospholipids, and adenine nucleotides (ATP, ADP, and AMP) (Hawkesford et al., 2012). In addition, P plays an important role in regulating enzymatic reactions and signalling processes through protein phosphorylation/dephosphorylation mechanisms (Hawkesford et al., 2012). Owing to its role in various physiological functions, the requirement of P in land plants is the fifth highest next to nitrogen, potassium, calcium, and magnesium; generally, the P concentration corresponds to approximately 0.2% of the dry matter of plants (Kirkby, 2012).

Land plants absorb P only in its inorganic phosphate (Pi) form present in the soil through their roots or mycorrhizae (Bieleski, 1973). Land plants possess two kinds of Pi transporters, namely low- and high-affinity transporters (Furihata et al., 1992; Muchhal et al., 1996; Kai et al., 1997; Leggewie et al., 1997; Liu et al., 1998), and their Michaelis-Menten constant (*Km*) values are estimated to be 50–100 µM and 2.5–12.3 µM *in planta,* respectively, based on radioactive Pi uptake experiments (Nussaume et al., 2011). These transporter proteins commonly harbour 12 transmembrane-spanning regions, with a large hydrophilic charged part dividing the protein molecules into two distinct domains containing six transmembrane regions in their structures. These transporter proteins transport one Pi with two to four protons (H+) into the root against the electrochemical gradient across the root surface (Ullrich-Eberius et al., 1981, 1984; Sakano, 1990; Mimura, 1999; Raghothama, 1999). In this process, the plasma membrane H+-ATPase contributes to generating the H+ electrochemical gradient and maintaining the cytosolic pH for Pi/H+ symport (Ullrich-Eberius et al., 1981, 1984; Mimura, 1999; Raghothama, 1999). The response to the change in Pi availability is sophisticated and well-regulated from the transcriptional to the post-translational level in land plants (Secco et al., 2012; Gu et al., 2016). Under low Pi availability, the expression of Pi transporter genes is activated by Pi-starvation-responsive transcription factors, such as the PHR protein-harbouring MYB domain and WRKY proteins (Rubio et al., 2001; Zhou et al., 2008; Gu et al., 2016). In addition, microRNA399 and microRNA827 support Pi uptake and accumulation in plants at the post-transcriptional step (Fujii et al., 2005; Aung et al.,2006; Bari et al., 2006; Chiou et al., 2006; Franco-Zorrilla et al., 2007; Lin et al., 2010; Secco et al., 2012; Gu et al., 2016). Simultaneously, a negative feedback system to suppress excessive Pi uptake is switched on, such as an increase in class 1 SPX domain-containing proteins, which suppress the expression of Pi-starvation responsive genes, and the non-protein coding gene *IPS1*, which acts as a target mimicry of microRNA399 under Pi-starvation conditions (Franso-Zorrilla et al., 2007; Wang et al., 2009; Liu et al., 2010; Secco et al., 2012; Puga et al., 2014; Wang et al., 2014). Under field conditions, we have to note that not only the enhancement of Pi uptake activity, but also the dissolution of fixed Pi in the soil, such as the exudation of organic acid from roots, is required to improve P use efficiency in land plants (Heuer et al., 2017). In contrast, under adequate Pi availability, Pi transporters are actively degraded by an E2 ubiquitin conjugase-related protein, PHO2, leading to the downregulation of Pi absorption (Delhaize & Randall, 1995; Dong et al., 1998; Aung et al., 2006; Bari et al., 2006).

After the absorption of Pi from the rhizosphere, it is transported to the shoot through the xylem by the transpiration stream and is mainly distributed in the mesophyll cells (Mimura, 1999). When Pi is sufficiently supplied to the mesophyll cells, it is stored in the vacuoles to maintain its concentration in the cytosol and organelles such as the chloroplasts and mitochondria (Mimura et al., 1990, 1992, 1996; Pratt et al., 2009). On the other hand, under Pi deficiency, Pi is exported from the vacuoles to the cytosol and is preferentially distributed into various organelles to maintain cellular physiological functions (Mimura et al., 1990, 1992, 1996; Pratt et al., 2009). These Pi homeostatic functions can be achieved by the interaction between the cytoplasm, apoplast, and vacuoles at the cellular level, and a preferential Pi distribution is also found among leaves depending on their age (Biddulph et al., 1958; Lee et al., 1990; Mimura, 1995, 1999; Mimura et al., 1996). Pi homeostatic functions are highly effective under Pi deficiency; however, the homeostatic functions under high Pi conditions are less effective because the equilibrium between Pi loading to the vacuole and Pi transportation from the xylem is dissociated in plant cells (Mimura et al., 1992). In fact, the apoplastic Pi concentration in leaf cells increases when the Pi application concentration exceeds a homeostatic capacity in the leaves (Mimura et al., 1990, 1992).

As mentioned above, although Pi is essential for plant growth, excess Pi application to plants leads to chlorosis and necrosis in the leaves. These phenomena have been recognized as P toxicity (Rossiter, 1952; Bhatti & Loneragan, 1970; Clarkson & Scattergood, 1982). To our knowledge, P toxicity was first reported in 1917 by John W. Shive (Shive, 1918). He examined the effect of different levels of P application on soybean growth using soil- and water-culture methods, concluding that excess Pi application causes specific injury in soybean leaves (Shive, 1918). Such toxicity symptoms have been observed in the leaves of various land plants, such as rice, wheat, barley, and *Arabidopsis* (Bhatti & Loneragan, 1970; Aung et al., 2006; Chiou et al., 2006; Wang et al., 2009; Liu et al., 2010). In general, P toxicity occurs when the Pi concentration exceeds approximately 1% of the leaf dry matter (Bhatti & Loneragan, 1970). Even in natural environments, P toxicity has also been observed. Specht (1963) showed that P fertilization in sandy soils, which have low Pi concentrations, triggered P toxicity symptoms in native plant species such as *Proteaceae* (ex. *Banksia* species) in South Australia. Several *Proteaceae* are known as P-sensitive plants compared with crop plants (Specht, 1963; Parks et al., 2000; Shane et al., 2004a). Actually, Hawkins et al. (2008) reported that *Proteaceae* plants of *Leucadendron* suffer from P toxicity when they are grown in post-agricultural lands in South Africa. It has been reported that this high P sensitivity is because *Proteaceae* species fail to downregulate Pi uptake under higher external Pi conditions; besides, these plants show a higher Pi translocation from the roots to the leaves because of the lower Pi storage capacity in the roots and stems than that in crop plants (Parks et al., 2000; Shane et al., 2004a, b; Shane and Lambers, 2006; de Campos et al., 2013). Interestingly, P toxicity symptoms were accelerated with higher calcium (Ca) application in *Proteaceae* (Nicholas and Beardsell, 1981). Recently, Hayes et al. (2019) proposed that Ca stimulates Pi loading to leaf mesophyll cells and enhances P toxicity symptoms. This suggests that the increased Pi concentration in leaf mesophyll cells is a primary cause of necrotic symptoms.

P toxicity has been assumed to be caused by zinc (Zn) deficiency in the leaves because the Zn concentration decreases depending on the dosage of Pi application in plants (Singh et al., 1988; Zhu et al., 2001; Zhang et al., 2002; Hawkins et al., 2008; Hawkesford et al., 2012). Several hypothetical mechanisms have been proposed to explain P-induced Zn deficiency in plants. Some of the major mechanisms include 1) the dilution effect of Zn on the tissue growth stimulated by P application, 2) inhibition of Zn absorption by roots under excess P application through mineral interaction in soil (Loneragan et al., 1979), 3) suppression of Zn translocation from the root to the shoot (Singh et al., 1988), and 4) inhibition of Zn acquisition depending on the mycorrhizae under excess P application (Ova et al., 2015). In contrast, several studies have shown that P toxicity is observed in plant leaves despite normal Zn accumulation in the leaves (Loneragan et al., 1979; Cakmak & Marschner, 1987; Lambers et al., 2002; Shane et al., 2004a; Ova et al., 2015; Hayes et al., 2019). Therefore, P toxicity cannot be explained only by the suppression of Zn acquisition from the soil to the leaves. Interestingly, Delhaize and Randall (1995) observed that light intensity directly modulates the P toxicity symptoms in the Pi-accumulating *Arabidopsis* mutant *pho2*, and limiting the illumination alleviates P toxicity. This observation implies the involvement of photosynthesis in the occurrence of P toxicity, but the detailed molecular mechanisms of P toxicity have not yet been addressed in land plants.

In this study, we investigated the detailed mechanisms of P toxicity that cause necrosis in land plants, especially in the leaves. Based on a previous study that showed the light requirement for emerging P toxicity in leaves (Delhaize and Randall, 1995), we hypothesized that the stimulation of reactive oxygen species (ROS) production by photosynthesis in chloroplasts would be the cause of chlorosis and necrosis symptoms in P toxicity. Here, we used rice plants and discussed the whole phenomenon of P toxicity in land plants.

**MATERIALS AND METHODS**

**Plant growth conditions**

*Oryza sativa* L. ‘Notohikari’ wild-type and Rubisco-sense and -antisense rice plants were used (Makino et al., 2000; Suzuki et al., 2007). Seeds were imbibed at 30 °C for three days and subsequently grown on a plastic net floating on tap water for three weeks in an environmentally controlled phytotron (NK system, Japan). Subsequently, rice seedlings were grown in a hydroponic culture (Makino et al., 1994). Here, we used six Piapplication conditions by changing the NaH2PO4 concentration: 0.06 (low-Pi), 0.6 (control-Pi), 1.2,1.8, 2.4, and 3.0 mM. The Na+ concentration was normalised by applying NaCl. The pH of the hydroponic culture was adjusted to 5.2 with 6 M HCl, and the solution was replenished twice in a week. The chamber was maintained at 60 % relative humidity with a 14-h light (28 oC) and a 10-h dark (25 oC) photoperiod. The light intensity was 500–600 µmol photons m−2 s−1. All physiological and genetic analyses were performed for fully and newly expanded leaves after 70 days of their germination.

**Chlorophyll and nitrogen concentration measurements**

The leaf chlorophyll (Chl) concentration and the Chl *a*/*b* ratio were determined as reported previously (Takagi et al., 2019). In brief, leaf segments were incubated in *N,N*-dimethylformamide at 4 °C overnight, and the absorbance values of the aliquot were measured at 750 nm, 663.8 nm, and 646.8 nm to calculate the chlorophyll concentration (Porra et al., 1989). The total leaf nitrogen (N) concentration was determined using Nessler’s reagent in a digestion solution after the addition of potassium sodium tartrate (KNaC4H4O6), and absorbance was measured at 420 nm (Takagi et al., 2017).

**Inorganic phosphate and organic phosphate quantifications**

Leaf Pi and organic phosphate (Po) concentrations were quantified by the molybdenum blue method according to Kurita et al. (2014).

**Measurements of photosynthetic activities**

Gas exchange analysis, Chl fluorescence, and P700+ were simultaneously measured using a combined system of GFS-3010 and Dual-PAM-100 (Heinz WalzGmbH, Germany). Ambient air (40 Pa CO2, 21 kPa O2) and gases with pure CO2 were mixed to determine the CO2 concentration during the measurements. The gases were saturated with water vapour at 18.0± 0.1 °C, and the leaf temperature was maintained at 25 °C. The following Chl fluorescence parameters were calculated as described by Baker (2008): *F*o, minimum fluorescence yield; *F*m, maximum fluorescence yield; *F*m’, maximum fluorescence yield under the illumination; and *F*s, steady-state fluorescence yield. A measuring light (0.1 µmol photons m–2 s–1) and a saturated pulse (20,000 µmol photons m–2 s–1, 600 ms) were applied to determine *F*o, *F*m, and *F*m’. The oxidation–reduction state of P700 in photosystem (PS) I was determined according to the method of Klughammer & Schreiber (1994). The maximum oxidation level of P700 (*P*m) was obtained using a saturated pulse under far-red light illumination. The oxidation–reduction state of P700 was determined using the oxidation level of P700 at the steady-state (*P*) and the maximum oxidation level of P700 under illumination (*P*m’). To determine the *proton motive force* (*pmf*), H+-efflux conductance (gH+), and H+-efflux rate (*V*H+) in leaves, the electrochromic shift (ECS) was measured using the dark-interval relaxation kinetics analysis (Sacksteder & Kramer, 2000). The value of *pmf* was normalised by the ECS signal obtained by a single turnover flash (10 µs) (Takagi et al., 2017). Actinic red light (AL) was used to measure the photosynthetic parameters.

**Inductively coupled plasma atomic emission spectroscopy (ICP-AES) measurements**

The leaf mineral concentration was determined according to Mekawy et al. (2018). Leaf blades were dried at 80 oC for one day and crushed using a µT-48 (TAITEC, Japan). The crushed samples were digested overnight in an acid mixture (HNO3:H2SO4:HClO4 = 5:1:2). Subsequently, the solution was heated at 150 oC for 30 min in a heat block. After cooling, the solutions were heated again at 200 oC for 1 h. The above process was continued until the solutions became colourless. The obtained solutions were used for ICP-AES analysis (iCAP6000; Thermo Fisher, Japan).

**Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis**

SDS-PAGE and western blotting were performed as previously described (Takagi et al., 2017). In brief, the extracted proteins were electrophoresed on 12.5% (w/v) polyacrylamide gels containing 0.1% (w/v) SDS and 6 M urea. Proteins were separated using SDS-PAGE, transferred onto polyvinylidene difluoride (PVDF) membranes and then blocked with skimmed milk for 30 min at 25°C. The PVDF membranes were subsequently incubated with a polyclonal antibody specific for Rubisco activase (Agrisera, Sweden) for 1 h at 25°C. The target proteins were detected using the SuperSignal™ West Dura Extended Duration Substrate kit (Thermo Fisher Scientific K.K., Japan) and LAS 4000 (Fuji film, Japan). The target protein concentration was determined using Image J software (National Institutes of Health, USA).

**Metabolite analysis**

Sugar phosphates and adenylates were extracted as previously described by Noguchi et al. (2018). Leaves were sampled under illumination at 1,100 µmol photons m–2 s–1 in ambient air (40 Pa CO2, 21 kPa O2, 25 oC), and immediately frozen in liquid N2. The frozen leaves were ground with a mortar and pestle in liquid N2. Methanol was added to the homogenised leaves, and the same volume of a solution containing internal standards (100 µM PIPES and 100 µM methionine sulfone) were mixed after vortexing. After centrifugation, the resulting supernatants were filtered through a 3 kDa cut-off filter (Millipore) at 16,100 × *g* at 4 oC for 30 min. Sugar phosphates and adenylates were separated by capillary electrophoresis triple quadrupole mass spectrometry (CE-QQQ-MS; 7100 Capillary Electrophoresis, MS; 6420 Triple Quad LC/MS, Agilent Technologies, USA) in multiple reaction monitoring mode as described previously (Miyagi et al., 2010, 2019). All CE-QQQ-MS data were processed using MassHunter software (Agilent Technologies).

**Rubisco assay**

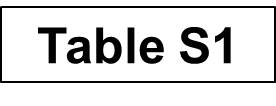
The Rubisco concentration in the leaf blades was determined as described by Makino et al. (1986). Rubisco was separated by SDS-PAGE and stained with Coomassie Brilliant Blue R-250 (CBB-R). Subsequently, the Rubisco protein bound to CBB-R was cut out from the SDS-PAGE gel and extracted with formamide at 50 oC for 5 h. The absorption of the extract was measured at 595 nm, and the Rubisco concentration was determined.

Rubisco activation was determined as described by Suganami et al. (2018). Leaf blades were illuminated at an irradiance of 1,100 µmol photons m–2 s–1 and 25 oC under ambient air conditions (40 Pa CO2, 21 kPa O2) for at least 1 h, and the illuminated leaf was immediately frozen in liquid N2. The frozen leaf was homogenised in the Rubisco extraction buffer [100 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) / NaOH (pH 8.0), 20 mM MgCl2, and 10 mM dithiothreitol (DTT)] with quartz sand on ice and centrifuged (19,000 × *g*, 10 s at 4 oC) to obtain a solution containing Rubisco. Rubisco activation was determined by multiplying the initial activity with the maximum activity, and the carbamylation potential was determined by multiplying the total activity with the maximum activity.

**Measurement of ascorbate peroxidase and superoxide dismutase activities**

Leaf blades were homogenised using an enzyme extract solution [50 mM potassium phosphate buffer (pH 7.0), 1 mM EDTA, 20% (w/v) sorbitol, 5% (w/v) polyvinylpyrrolidone, 1% (w/v) Tween-20, and 1 mM ascorbate] with quartz sand. The ascorbate peroxidase (APX) activities were measured following Amako et al. (1994), and the superoxide dismutase (SOD) activities were measured according to Flóhe & Ötting (1984).

**Real-time PCR analysis for mRNA quantification**

mRNA was purified from rice leaf blades as described by Suzuki et al. (2004). Total RNA was quantified by measuring the absorbance at 260 nm. Real-time PCR analysis was conducted using the StepOnePlus™ Real-Time PCR System (Applied Biosystems, USA) and Fast SYBR Green Master Mix (Life Technologies Japan) (Ogawa et al., 2012). The primers used for mRNA expression analysis are listed in Table S1.

**Quantification of lipid hydroperoxide**

Lipid hydroperoxide (LOOH) in rice leaf blades was quantified using Spy-LHP (Dojindo, Japan). Leaf blades were homogenised in the extraction buffer [Potassium-phosphate buffer (pH 7.0), 1 mM EDTA, 12.5% (v/v) glycerol], and the resulting leaf extract was incubated with 2.7 µM Spy-LHP for 30 min on ice. After centrifugation, the supernatant was monitored for fluorescence emission (RF-1500; Shimazu). The excitation and emission wavelengths were 520 and 540 nm, respectively.

**Quantification of phytic acid**

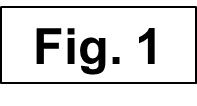
The phytic acid concentration was measured as described by Perera et al. (2019). Fully expanded leaves were homogenised in 0.66 M HCl with quartz sand, and phytic acid was extracted by shaking the homogenised solution at 25 oC for 17 h. The extracts were centrifuged (19,000 × *g*, 10 min at 4 oC) and the supernatant was neutralised with 0.75 M NaOH. The phytic acid concentration was measured using the Phytic Acid (Total Phosphorus) Assay Kit (Megazyme International Ireland Ltd., Ireland).

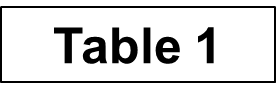
**Statistical analysis**

All measurement data were expressed as the mean ± SD of at least three independent biological analyses. To detect the differences among the rice plants grown under different Pi conditions or rice mutants, we used one-way analysis of variance (ANOVA) and Tukey’s honest significant difference (HSD) test. All statistical analyses were performed using Origin Pro 2019 software (LightStone Corp., Japan).

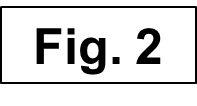
**RESULTS**

**Effects of Pi application and P toxicity on rice plants**

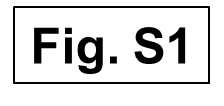
To investigate the effects of Pi application, wild-type (WT) rice plants were grown under six Pi levels ranging from 0.06 to 3.0 mM (Figure 1). Compared with the low-Pi and control-Pi treatments (0.06 and 0.6 mM), higher Pi application (above 1.2 mM) caused chlorosis and necrosis at the tip of the leaf blade (Figure 1a). Such a phenomenon has previously been observed in rice plants that accumulate excess Pi (Aung et al., 2006; Wang et al., 2009; Liu et al., 2010). The plant height was not affected by Pi application (Figure 1b). The Chl concentration of the leaf blades was the highest in plants treated with 0.06 mM Pi and decreased gradually with increasing Pi application (Figure 1c). The total dry weight, including roots, was reduced in the 0.06 mM Pi treatments, and Pi applications higher than 1.2 mM further decreased the dry weight (Figure 1d). The leaf sheath weight was the most susceptible to an increase in Pi application, and Pi levels higher than 1.8 mM significantly decreased the leaf sheath weight compared to that in the control-Pi treatments (Figure 1d).

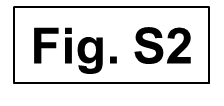
To confirm whether different Pi application levels affected the leaf P concentration, the Pi and Po concentration in the leaf blades were determined per unit of fresh weight (F.W.) and leaf area. The Pi concentration increased with increasing Pi application, and the Po concentration also increased, but to a lesser extent (Table 1). These results indicate that Pi was accumulated in the leaves, depending on Pi concentration, and P toxicity was caused in WT rice plants growing under higher Pi conditions than that in the control-Pi conditions.

**Excessive Pi application declines the Rubisco-limited photosynthesis**

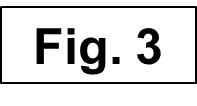
Because the onset of P toxicity symptoms requires illumination, photosynthetic metabolism should provide an insight into the cause of P toxicity under illumination conditions. We measured the CO2 assimilation rate (*A*) and quantum yield of photosystem (PS) II [Y(II)], for dissipation by downregulation [Y(NPQ)], and other non-photochemical losses [Y(NO)] (Kramer et al., 2004), as a function of the internal partial pressures of CO2 in the leaves (Ci) of rice grown under different Pi conditions. The low-Pi plants showed lower *A* than did the control-Pi plants, especially at high Ci levels (Figure 2a). The Y(II) was lower in the low-Pi plants than in the control-Pi plants at high Ci levels (Figure 2b). In contrast, the low-Pi plants showed higher Y(NPQ) than did the control-Pi plants at high Ci levels (Figure 2c). The low-Pi and control-Pi plants showed similar Y(NO) values (Figure 2d). These responses showed a typical Pi limitation in photosynthesis (Sharkey, 1985; Fabre et al., 2019); and lowering the Pi decreased *A* by limiting the Pi turnover, and NPQ was stimulated to protect the photosynthetic electron transport (PET) system at high Ci levels.

On the other hand, *A* decreased with an increase in Pi application to more than 1.2 mM Pi (Figure 2e). A decrease in the CO2 assimilation rate was also observed in *Proteaceae* (*Hakea prostrata*) grown under high Pi conditions (Shane et al., 2004a). In addition, the response of *A* to Ci became more linear. The initial slope of *A:*Ci was the steepest in the control-Pi plants and decreased with increasing Pi application (Figure 2i). Because the initial slope of *A*:Ci suggested Rubisco activity (von Caemmerer & Farquhar, 1981), these results indicate that the Rubisco activity decreased with increasing Pi application. An increase in Pi application lowered the Y(II) in a wide range of Ci levels, and the response of Y(II) to Ci also became linear in a manner similar to that of *A* (Figure 2f). The Y(NPQ) increased with an increase in Pi application, but was suppressed at lower Ci levels in the 3.0 mM Pi plants than in the control-Pi plants (Figure 2g). The Y(NO) was similar in all Pi applications, except for the 3.0 mM Pi conditions (Figure 2h). This implies that although NPQ was stimulated with increasing Pi accumulation in the leaves, excessive Pi accumulation disturbed the redox state of the PET chain. In fact, the *F*v/*F*m value was significantly decreased in the 3.0 mM Pi treatments (Figure 2j).

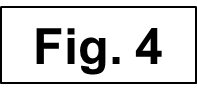
To determine the detailed effects of excessive Pi application on photosynthesis, we also examined the PSI parameters [Y(I), Y(ND), and Y(NA)] and H+-management within the chloroplasts [*proton motive force* (*pmf*), H+-conductance in chloroplastic ATPase (gH+) and H+-efflux rate in chloroplastic ATPase (*V*H+)] in plants grown under low-Pi, control-Pi, and 3.0 mM Pi conditions. The Y(I) indicates the photochemical quenching in PSI, whereas the Y(ND) and Y(NA) indicate the PET limitations at the donor and acceptor sides of PSI, respectively (Klughammer & Schreiber, 1994). The Y(I) showed a response similar to that of the Y(II) (Figure S1a). The Y(ND) decreased with increasing Ci levels both under low-Pi and control-Pi conditions (Figure S1b). The Y(ND) in the 3.0 mM Pi plants decreased with an increase in Ci, but the values were higher than those in the low-Pi and control-Pi plants (Figure S1b). The Y(NA) increased with increasing Ci in both low-Pi and control-Pi plants, but the Y(NA) was higher in the low-Pi plants than in the control-Pi plants (Figure S1c). In contrast, the 3.0 mM Pi plants showed the highest Y(NA) at low Ci levels, and the Y(NA) was maintained at higher levels in the 3.0 mM Pi plants than in the control-Pi plants (Figure S1c). These results indicate that the low-Pi plants reduced PSI at higher Ci level with photosynthesis limitation, but the 3.0 mM Pi plants caused a disturbance in the redox state of PSI, shifting PSI to a more reduced state, despite inducing the donor-side PET limitation in PSI.

The *pmf* and gH+ were similar between the control-Pi and low-Pi plants, but *V*H+ was slightly lower in the low-Pi plants than in the control-Pi plants (Figure S2). In contrast, the 3.0 mM Pi plants showed lower *pmf*, gH+, and *V*H+ than the control-Pi plants (Figure S2). Both the gH+ and *VH+* showed linear responses to Ci in a manner similar to *A*, Y(II), and Y(I) (Figure 2e, f and Figure S1a). These results indicate that both *pmf* generation by PET reaction and ATP synthesis were suppressed under 3.0 mM Pi conditions (Takagi et al., 2016b, 2017). From these observations, we found that excessive Pi accumulation suppressed photosynthesis over a wide range of Ci levels; this phenomenon was different from that caused by low-Pi application in rice plants.

**Excessive Pi application increases the sugar phosphate concentration in both the chloroplasts and cytosol**

To evaluate the effects of excessive Pi application on sugar phosphate metabolism, we analysed the concentration of major sugar phosphates and adenylates in leaves under illumination conditions (see Materials and Methods). In the low-Pi plants, a large number of sugar phosphates were decreased compared to that in the control-Pi plants (Figure 3b). However, ribose-5-phosphate (R5P) showed similar levels in both the low-Pi and control-Pi plants, and the levels of ribulose-5-phosphate (Ru5P) and glycerol-3-phosphate (G-3-P) were higher in the low-Pi plants than in the control-Pi plants. The total adenylate concentration was lower in the low-Pi plants than in the control-Pi plants, and the ATP/ADP ratio was also decreased (Figure 3c, d). These results indicate that the substrates for photosynthesis in the Calvin cycle decreased, and that the ATP consumption exceeded ATP synthesis, owing to the lower adenylate concentration in low-Pi plants than in control-Pi plants. These observations were clearly consistent with the photosynthetic kinetics in low-Pi plants (Figure 2a) (Sharkey, 1985; Fabre et al., 2019). The increases in Ru5P and R5P levels in low-Pi plants might be caused by the limited ATP supply (Figure 3a, b). The increase in G-3-P has been observed under P deficiency for remodelling lipid composition (Cheng et al., 2011; Nakamura, 2013); that is, an increase in G-3-P represents the P starvation response (Figure 3b). An increase in Pi application increased the sugar phosphate concentration (Figure 3b). In particular, the levels of glycelaldehyde 3-phosphate (GA3P), dihydroxyacetone phosphate (DHAP), fructose-6-phosphate (F-6-P), 6-phosphogluconate (6-PG), glucose-1-phosphate (G-1-P), and glucose-6-phosphate (G-6-P) increased with increasing Pi application (Figure 3b). The total adenylate levels also increased with increasing Pi application (Figure 3c). In contrast, the ATP/ADP ratio was enhanced with increasing Pi application (Figure 3d). These results indicate that ATP consumption was suppressed more than ATP synthesis under high Pi application. This observation clearly matched the decrease in H+-conductance of the chloroplastic ATP synthase, as indicated by the gH+ value (Figure S2b).

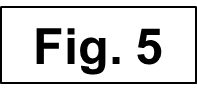
**Excessive Pi application does not affect the concentration of Rubisco, but does decrease Rubisco activation**

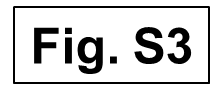
The total leaf N concentration significantly decreased in the low-Pi treatments and was almost constant in Pi treatments of more than 1.2 mM (Figure 4a). Rubisco concentration showed a similar response to the total leaf N concentration. A low-Pi application slightly decreased the Rubisco concentration, but a higher Pi application did not change it (Figure 4b).

The Rubisco activation was high in the low-Pi treatments, but decreased with increasing Pi application (Figure 4c). The carbamylation potential of Rubisco, which is lowered by a tight binging inhibitor, such as 2-carboxy-D-arabinitol 1-phosphate, did not differ among different levels of Pi application (Figure 4d). Indeed, the Rubisco in the night-sampled leaves showed significantly lower activation and carbamylation potential compared with the Rubisco sampled under illumination (Figure 4c, d). Thus, one of the reasons for the decline in photosynthesis due to excessive Pi application is the decline in *in vivo* Rubisco activation.

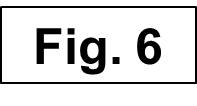
Because Rubisco activation is catalysed by Rubisco activase (RCA) (Salvucci et al., 1985), we examined the RCA concentration of the leaves. Three isoforms of RCA resulting from alternative splicing and limited proteolysis have been reported in rice (Portis 2003; Vargas-Suárez et al., 2004; Fukayama et al., 2012). According to Fukayama et al. (2012), an upper protein band corresponds to the *α-*form of RCA, which has redox-active cysteine residues; a middle protein band corresponds to the *β*-form, which lacks redox-active Cys residues, and a lower protein band corresponds to the N-terminal processed *β* (*β\**)-form in the leaves (Figure 4e). The total amount of RCA, including the *α-*, *β-,* and *β\*-*forms, was similar among different Pi treatments (Figure 4f). However, a high Pi application decreased the concentrations of *α*- and *β*-forms compared to those in the control-Pi conditions (Figure 4g). Contrary to the change in the *α-* and *β*-forms, the *β\**-form concentration increased with increasing Pi application (Figure 4g). When the isoform ratios of *α*/*β* and *β*\*/*β* were calculated, we found that an increase in Pi application decreased the *α*/*β* ratio and increased the *β*\*/*β* ratio compared to those in the low-Pi and control plants (Figure 4h, i). These results indicate that an increase in Pi application modified the RCA isoform composition of the leaves.

**Excessive Pi application stimulates APX activity but suppresses Cu/Zn-SOD activities**

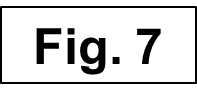
As shown in Figure 2 and Supplemental Figures S1 and S2, an increase in Pi application limited photosynthesis and reduced the PET chain in the leaves. These results imply that ROS production was stimulated under P toxicity conditions (Cakmak, 2005; Takagi et al., 2016a). Based on this consideration, ascorbate peroxidase (APX) and superoxide dismutase (SOD) activities were measured as key candidates for ROS-scavenging enzymes. The total APX activities, including the cytosolic and chloroplastic APX activities, tended to be higher in the 2.4 and 3.0 mM Pi treatments than in the other Pi treatments (Figure 5a). The cytosolic APX activity did not differ among the Pi treatments, but the chloroplastic APX activity was significantly increased in the 3.0 mM Pi treatments (Figure 5a). The increase in chloroplastic APX activity in the 3.0 mM Pi treatments was also observed on a leaf Chl basis (Figure 5b). In contrast, a higher Pi application significantly decreased the total SOD activities, including the Fe-, Mn-, and Cu/Zn-SOD activities (Figure 5c). A decrease in the total SOD activities under excessive Pi application was also reported by Cakmak and Marschner (1987). The decrease in total SOD activities was due to a significant decrease in the Cu/Zn-SOD activities rather than a decrease in the Fe- and Mn-SOD activities (Figure 5c).

To understand the general response of APX and SOD activities under the limitation of photosynthesis, the APX and SOD activities were measured in the Rubisco-sense and -antisense plants (Figure S3a) (Makino et al., 2000; Suzuki et al., 2007). Under control-Pi conditions, the Rubisco-antisense plants showed significantly higher total and chloroplastic APX activities than did the WT and Rubisco-sense plants, although the cytosolic APX activity was similar among them (Figure S3b, c). Under 3.0 mM Pi conditions, the chloroplastic APX activities in WT and Rubisco-sense plants were more activated than those under the control-Pi conditions, and showed similar values to those in Rubisco-antisense plants (Figure S3b, c). The total- and Cu/Zn-SOD activities were higher in the Rubisco-antisense plants than in the WT and Rubisco-sense plants under the control-Pi conditions (Figure S3d). However, under 3.0 mM Pi conditions, the Cu/Zn-SOD activities in the Rubisco-antisense plants were suppressed similar to those in the WT and Rubisco-sense plants. These results indicate that the P toxicity-mediated suppression of photosynthesis triggered an increase in APX activity similar to that in the Rubisco-antisense plants, but the Cu/Zn-SOD activities showed different responses between excessive Pi treatments and decreased Rubisco concentration, even though both situations limit photosynthesis.

**Higher Pi application does not suppress all Cu/Zn-SOD mRNA expressions**

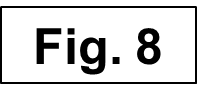
Rice plants have five Cu/Zn-SOD genes in their genomes (RiceXPro; http://ricexpro.dna.affrc.go.jp/). All mRNA expression levels of Cu/Zn-SOD were determined on a leaf F.W. and a 18S ribosome mRNA expression basis relative to those of the control-Pi plants, respectively. The expressions of *cytCu/ZnSOD1,2* and *CuZnSOD3* increased with increasing Pi application (Figure 6a-c), but those of *perCuZnSOD* and *chlCuZnSOD* were not affected by Pi application (Figure 6d, e). Thus, the responses of these mRNA expressions did not account for the decrease in Cu/Zn-SOD activities.

**Excessive Pi application does not inhibit the absorption of other nutrients, but might decrease nutrient availabilities**

To investigate why the Cu/Zn-SOD activities decreased in the higher Pi treatments, the concentrations of several essential nutrient elements were determined. The P concentration increased with increasing Pi application (Figure **7a). The concentrations values of K, Ca, and Fe were not affected by Pi application (Figure 7a), but those of Mg, Mn, Zn, and Cu increased with increasing Pi application (Figure 7a).

To determine the nutrient availability within leaves, we next examined the mRNA expressions of several metal deficiency-responsive genes, such as *ZIP4* (Ishimaru et al., 2005), *IRO2* (Ogo et al., 2006) and *COPT1* (Yuan et al., 2011), which are expressed under Zn-, Fe-, and Cu-deficient conditions, respectively. The *ZIP4* and *IRO2* expressions increased with increasing Pi application (Figure 7b); *COPT1* expression showed no significant change with varying Pi application (Figure 7b). These results suggest that excessive Pi application did not inhibit metal transport to leaves, but Zn- and Fe-deficiency occurred within cells under excessive Pi application.

**Phytic acid synthesis is upregulated by excessive Pi application**

Mitsuhashi et al. (2005) reported that exposing cultured cells of *Catharanthus roseus* and *Arabidopsis* to high Pi conditions stimulates phytic acid synthesis and the accumulation of phytic acid in both the cytosol and vacuoles. Based on this observation, we examined whether high Pi accumulation stimulates phytic acid synthesis in leaves. Perera et al. (2018) summarised the putative genes involved in phytic acid synthesis in rice. Among these, we selected those that are expressed in leaves using the Rice-XPro database and quantified their mRNA expression. Figure 8a shows the phytic acid synthesis pathways as well as the genes involved in these pathways (Suzuki et al., 2007; Perera et al., 2018). The *INO1, IPK1, IPK2, 2-PGK, ITPK3-1, ITPK3-2, ITPK5,* and *ITPK6* transcript levels tended to increase with increasing Pi application (Figure 8b). In contrast, the *IMP1-1* transcript levels increased only under low-Pi conditions. The *IMP1-2*, *ITPK1,* and *ITPK2* transcript levels showed no clear responses to Pi application.

To confirm that the change in mRNA expression of the genes involved in phytic acid synthesis actually changed the phytic acid concentration in leaves, we quantified the phytic acid concentration in the leaves. The leaf phytic acid concentration was similar in low-Pi and control-Pi plants (Figure 8c, d). As with the increase in Pi application, the leaf phytic acid concentration increased and the 2.4 and 3.0 mM Pi plants showed significantly higher phytic acid concentrations than did the control-Pi plants (Figure 8c, d). Figure 8e shows the phytic acid/free Pi ratio in the leaves. Under 0.06 mM Pi conditions, the phytic acid/free Pi ratio was significantly higher than that under other Pi applications. In contrast, the increase in Pi application from the control-Pi to 3.0 mM Pi did not change the phytic acid/free Pi ratio in the leaves. These results indicate that the phytic acid concentration increased with increasing Pi concentrations in the leaves of all Pi-treated plants, except for the low-Pi plants.

**DISCUSSION**

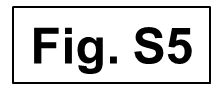
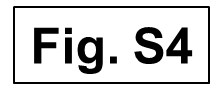
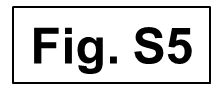
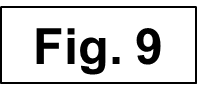
Owing to the low concentrations of Pi in the soil (on average 0.09 ppm [< 10 µM]; Pierre and Parker, 1927), Pi acquisition often limits plant growth and crop yield. Therefore, improving Pi absorption and utilisation in land plants is one of the targets for increasing agricultural productivity (Gu et al., 2016). In contrast, excessive Pi absorption in land plants causes P toxicity (Shive, 1918; Specht, 1963; Delhaize and Randall, 1995; Parks et al., 2000; Shane et al., 2004a, b; Hawkins et al., 2008; de Campos et al., 2013; Hayes et al., 2019). Therefore, to improve Pi-use efficiency in land plants, P toxicity must be avoided, and the mechanisms of P toxicity should be understood. However, the detailed mechanisms of illumination-dependent P toxicity that cause leaf chlorosis and necrosis in land plants remain to be clarified (Cakmak & Marscher, 1987; Delhaize & Randall, 1995; Ova et al., 2015). To address this question, we characterised the effects of different Pi application rates on rice plants from the aspect of photosynthesis. Here, we propose three important factors underlying P toxicity symptoms: the activation of the phytic acid synthesis pathway in the leaves, the limitation of photosynthesis, and the disruption of Cu/Zn-SOD activities.

We suggest that P toxicity is triggered by the accumulation of phytic acid in leaves. In seeds, phytic acid synthesis is stimulated in response to Pi accumulation for storing P and metals (Hawkesford et al., 2012; Su et al., 2018). In line with this observation, we found that phytic acid synthesis occurs substantially with an increase in Pi accumulation in the leaves due to the upregulated mRNA expression of genes involved in phytic acid synthesis (Table 1; Figure 8). The activation of phytic acid synthesis would be triggered by an increase in G6P concentration in the cytosol (Figure. 3b). Previous studies have shown that phytic acid is synthesised in the cytosol and is stored in the vacuoles of the leaf cells (Mitsuhashi et al., 2005; Nagy et al., 2009; Lee et al., 2015). Moreover, the suppression of Pi transport to vacuoles stimulates the symptoms of P toxicity (Liu et al., 2015; Liu et al., 2016). Our results showed that the sugar phosphate concentration increased in both the chloroplasts and cytosols under higher Pi application conditions (Figure. 3b). The triose phosphate translocator (TPT) exports 3-PGA and triose phosphate between the chloroplasts and the cytosol by exchanging Pi (Flügge & Heldt, 1984). An increase in the cytosolic Pi level would stimulate the export of triose phosphate from the chloroplasts to the cytosol (Flügge, 1992), as well as the metabolic flux involving sugar phosphates (Figure 3a, b). Phytic acid synthesis occurs via two distinct pathways (lipid-dependent and lipid-independent) which are both initiated by G6P (Figure 8a) (Suzuki et al., 2007; Perera et al., 2018). We found that the lipid-independent pathway responded to an increase in Pi accumulation in the leaves (Figure 8b), and a stable phytic acid/free Pi ratio from the control-Pi to 3.0 mM Pi conditions suggests that a certain proportion of Pi is converted to phytic acid in accordance with the sugar phosphate accumulation in the cytosol via the lipid-independent pathway (Figure 3b; Figure 8b-e).

An increase in the cytosolic Pi accelerates triose phosphate export and G6P synthesis, thereby stimulating the synthesis of phytic acid, which, in turn, precipitates Zn in the cytosol. Phytic acid has high metal-chelating activity, and Maenz et al. (1999) showed that Zn, rather than other metals, is an exclusive target of phytic acid. Furthermore, the cytosolic pH (around 7.5) is optimal for phytic acid to chelate Zn (Maenz et al., 1999; Mimura et al., 2000). From the perspective of thermodynamics, Pi can easily bind to divalent cations, including Zn2+ (Childs, 1970); however, phytic acid would further accelerate the unavailability of Zn within plant cells. Under the cytosolic pH, the major form of Pi corresponds to H2PO4- and HPO42-, and Zn exists in its aqueous form (Zn2+) (Krężel & Maret, 2016). Under such conditions, the thermodynamic equilibrium constant (logK) value between Zn2+ and H2PO4- for producing ZnH2PO4+ is approximately 1.2, and that between Zn2+ and HPO42- for producing ZnHPO4 is approximately 2.4 (Childs, 1970; Krężel & Maret, 2016). In contrast, phytic acid showed a much higher logK value for binding to Zn than did Pi, and the logK value reaches approximately 10.4 (calculated from the data in Krężel & Maret, 2016). This means that although the phytic acid concentration is much lower than Pi concentrations in the cytosols, the increase in phytic acid concentration could accelerate a binding reaction with Zn. At this time, we have to acknowledge the possibility that large amounts of Pi also stimulate the Zn precipitation because of the lack of Zn phosphate salt species found within cells. However, it is noteworthy that Pi showed a higher affinity (log K value) to Cu2+ than to Zn2+ (Childs, 1970). When we focused on *COPT1*, no significant response to the change in Pi application was found in the leaves (Figure 7b). From these considerations, we propose that the increase in phytic acid contributes to precipitating Zn within the cells under P toxicity conditions. In fact, Cakmak and Marschner (1987) observed that Pi accumulation increases the insoluble Zn concentration in leaves. This would be a cause of the increase in *ZIP4* expression and the decrease in Cu/Zn-SOD activities owing to the lower Zn availability within cells (Figures 5c and 7b). This can explain the previous observation of P toxicity symptoms similar to Zn deficiency symptoms in land plants, despite the substantial accumulation of Zn in leaves (Cakmak & Marscher, 1987; Shane et al., 2004a; Hawkins et al. 2008; Ova et al., 2015; Hayes et al., 2019). Fe is also a target of phytic acid accumulation (Maenz et al., 1999). In fact, the *IRO2* expression increased with increasing Pi accumulation (Figure 7b). However, decreases in the Fe- and Mn-SOD activities was not observed (Figure 5c). APX also contains Fe as a co-factor, and its activity decreases under Fe-deficient conditions (Ranieri et al., 2001). However, the APX activity increased under high Pi conditions (Figure 5a, b and Figure S3b, c). These results suggest that Fe-deficiency is less severe than Zn deficiency within cells under P toxicity conditions.

Is the phytic acid just a storage form of P or a toxic compound to stimulate P toxicity in plant leaves? Interestingly, the *IMP1-1* expression was activated under low-Pi conditions (Figure 8b). The phytic acid/free Pi ratio showed a response similar to that of *IMP1-1* expression (Figure 8e). These results suggest that the lipid-dependent phytic acid synthesis pathway could be activated under low-Pi conditions. Although phytic acid is a major storage form of Pi in seeds (Hawkesford et al., 2012), it has a physiological importance in plant physiology. For example, the inhibition of phytic acid synthesis leads to leaf morphology distortion in *Arabidopsis* or growth retardation in crop plants(Raboy et al., 2000; Stevenson-Paulik et al., 2005). Lee et al. (2015) demonstrated that phytic acid is required for activating LOS4/Gle1-mediated mRNA export from the nucleus in *Arabidopsis*, in a manner similarly to in animals and yeast, and it was also reported that the phytic acid deficiency causes mRNA accumulation within nuclei. In addition, phytic acid inactivates inward K+ conductance by mobilising Ca2+ in the plasma membranes in response to abscisic acid in the guard cells, contributing to pathogen defence (Lemtiri-Chlieh et al., 2000, 2003; Murphy et al., 2008; Nagy et al., 2009). Based on these studies, the lipid-dependent pathway might secure phytic acid synthesis under low-Pi availability to maintain phytic acid-dependent physiological reactions. Further investigation is required to confirm the validity of this hypothesis. In contrast, the stimulation of phytic acid synthesis under higher Pi conditions would also play some physiological roles. Under low-Pi availability, land plants develop root systems, such as root hairs or cluster roots, and the root biomass decreases with increasing Pi availability (Skene, 1998; Shane et al., 2004a, b; Shane et al., 2006; White, 2012; de Campos et al., 2013; Hayes et al., 2019). Stevenson-Paulik et al. (2005) reported that the *IPK1* mutant in *Arabidopsis,* which is suppressed phytic acid synthesis, responded less to the change in Pi availability and showed longer root hair than did the WT under high Pi conditions. As suggested by Stevenson-Paulik et al. (2005), the increase in phytic acid could contribute to sensing high Pi conditions. Based on this study, we suggest that a small amount of phytic acid can act as a signalling or regulator molecule, but excessive or unregulated phytic acid synthesis causes toxic effects in land plants.

The limitation of photosynthesis is enhanced by a decrease in RCA concentration. We found that Pi accumulation decreases electron sink capacities by deactivating Rubisco, but not lowering Rubisco concentration (Figures 2 and 4). Furthermore, the *α*- and *β*-forms of RCA significantly decreased with increasing Pi application (Figure 4e-i). Among the three RCA isoforms, the *α*- and *β*-forms are the major components of Rubisco activation (Zhang et al., 2002; Portis, 2003); it has not been addressed whether the *β\**-form has the ability to activate Rubisco. These results suggest that the target of P toxicity exists not in Rubisco itself, but within the isoforms of RCA, especially in the *α*- and *β*-forms. In the RCA mutants of *Arabidopsis* and rice, *A* linearly increased with increasing Ci levels in a manner similar to our results in WT plants under high Pi conditions (Zhang et al., 2002; Masumoto et al., 2012; Yamori et al., 2012). Although we could not elucidate the detailed mechanism to differentiate the concentration of RCA isoforms in the leaves, Vargas-Suárez et al. (2004) suggested that the *β\**-form is generated by limited proteolysis under stress conditions, such as drought, and changing the *β-*form to the *β\*-*form results in a higher chaperone activity. From this point, we suggest the possibility that the *β\**-form is upregulated to protect proteins under the oxidative stress conditions caused by P toxicity at the cost of Rubisco activation. Supporting this idea, Rokka et al. (2001) suggested that RCA plays a secondary role as a chaperone for protecting thylakoid membrane proteins under heat stress conditions; that is, the physiological functions of RCA would not be limited to the activation of Rubisco. We also suggest that the severe limitation of photosynthesis is caused by additional factors in concert with a decrease in RCA concentration. Badger & Lorimer (1981) reported that Pi acts as a weak Rubisco inhibitor *in vitro*; that is, Rubisco activation might be further reduced under P toxicity conditions *in vivo*. Moreover, Suzuki et al. (2012) reported that when only Rubisco severely limits the photosynthetic metabolic flux in the leaves, the ribulose 1,5-bisphosphate (RuBP) concentration is increased. However, under high Pi conditions, the GA3P and DHAP concentrations were higher than that of RuBP (Figure 3b). This result suggests that, in addition to RCA, another molecule should be suppressed under P toxicity conditions.

Under P toxicity conditions, leaf necrosis would be caused by oxidative stress resulting from the disruption of Cu/Zn-SOD activities, as well as photosynthetic limitation. When the PET activity exceeds the electron sink capacity, ROS production is stimulated in both PSII and PSI (Krieger-Liszkay, 2005; Sonoike, 2011; Pospíšil, 2016; Takagi et al., 2016a). ROS show high reactivity to biomolecules, including DNAs, proteins, and lipids; therefore, the accumulation of ROS triggers cellular dysfunction and causes cause cell death (Apel & Hirt, 2004). Under high Pi conditions, an increase was observed in the values of Y(NO) and Y(NA), which indicates the reduced states of PSII and PSI, respectively, despite an increase in the values of NPQ and Y(ND), which contributes to the suppression of ROS production both in PSII and PSI (Figure 2; Figure S1) (Müller et al., 2001; Takagi et al., 2017). Wada et al. (2018) reported that Rubisco-antisense plants show an over-reduction of the PET chain. Furthermore, Takagi et al. (2016b) reported that the suppression of electron sink activities inhibits both the PET reaction and *pmf* generation. At the same time, the values of gH+ and *V*H+ also decreased, because of the decrease in the ATP requirement of electron sink activities (Takagi et al., 2016b). Here, we observed that the ATP/ADP ratio significantly increased with increasing Pi application (Figure 3d). Moreover, *pmf*, gH+, and *V*H+ decreased under 3.0 mM Pi conditions (Figure S2). Therefore, a high Pi accumulation would cause an over-reduction of the PET chain, owing to a decrease in electron sink activities, including Rubisco deactivation. An increase in APX activity is in accordance with the stimulation of oxidative stress under P toxicity conditions, because chloroplastic APX activity responds to oxidative stress (Figure 5a, b) (Cakmak, 2005). Furthermore, the decrease in the Chl concentration with decreasing PSI (Figure 1c; Figure S4), as well as the decrease in *F*v/*F*m is in accordance with oxidative stress on the thylakoid membranes under excessive Pi application (Figure 2j) (Terashima et al., 1994; Sonoike, 2011; Takagi et al., 2016a). In addition, we found an increase in the expression of the ROS-responsive gene *PR5* (Ganesan & Thomas, 2001) and lipid hydroperoxide concentration (LOOH) under high Pi application (Figure S5a, c). In contrast, the expression of the programmed cell death-related gene *VPE2* (Deng et al., 2011) decreased under high Pi conditions (Figure S5b). Based on the illumination dependency of P toxicity symptoms (Delhaize & Randall, 1995), necrosis, but not programmed cell death is triggered by ROS production in thylakoid membranes; this would be intensified by both the suppression of Cu/Zn-SOD activities and the decrease in photosynthetic electron sink capacities (Figure 9).

In this present study, we discussed a detailed mechanism for P toxicity in rice (Figure 9). To date, much attention has been paid to phytic acid synthesis in seeds. However, our results indicated that maintaining an appropriate leaf phytic acid concentration is important for maintaining plant growth and escaping oxidative stress triggered by the PET reaction. Because the phytic acid synthesis pathway is activated by an increase in the cytosolic Pi concentration through an increase in the sugar phosphate concentration, the fine-tuning of Pi compartmentation within cells or modulation of sugar phosphate metabolic flux might contribute to improving the Pi-use efficiency in the absence of P toxicity symptoms. The present results would open new opportunities to design breeding strategies to improve P-use efficiency in crop plants.

**REFERENCE**

Amako, K., Chen, G.X. & Asada, K. (1994). Separate assays specific for ascorbate peroxidase and guaiacol peroxidase and for the chloroplastic and cytosolic isozymes of ascorbate peroxidase in plants. *Plant and Cell Physiology,* 35(3), 497-504.

Apel, K. & Hirt, H. (2004). Reactive oxygen species: metabolism, oxidative stress, and signal transduction. *Annual Review of Plant Biology*, 55, 373-399.

Aung, K., Lin, S.I., Wu, C.C., Huang, Y.T., Su, C.L., & Chiou, T.J. (2006). *pho2*, a phosphate overaccumulator, is caused by a nonsense mutation in a microRNA399 target gene. *Plant Physiology*, 141(3), 1000-1011.

Badger, M.R. & Lorimer, G.H. (1981). Interaction of sugar phosphates with the catalytic site of ribulose-1, 5-bisphosphate carboxylase. *Biochemistry*, 20(8), 2219-2225.

Baker, N.R. (2008). Chlorophyll fluorescence: a probe of photosynthesis in vivo. *Annual Review of Plant Biology*, *59*, 89-113.

Bari, R., Pant, B.D., Stitt, M. & Scheible, W.R. (2006). PHO2, microRNA399, and PHR1 define a phosphate-signaling pathway in plants. *Plant Physiology,* 141(3), 988-999.

Bhatti, A.S. & Loneragan, J.F. (1970). The effect of early superphosphate toxicity on the subsequent growth of wheat. *Australian Journal of Agricultural Research,* 21(6), 881-892.

Biddulph, O., Biddulph, S., Cory, R. & Koontz, H. (1958). Circulation patterns for phosphorus, sulfur and calcium in the bean plant. *Plant Physiology*, 33(4), 293-300.

Bieleski, R.L. (1973). Phosphate pools, phosphate transport, and phosphate availability. *Annual Review of Plant Physiology,* 24, 225-252.

Cakmak, I. (2005). The role of potassium in alleviating detrimental effects of abiotic stresses in plants. *Journal of Plant Nutrition and Soil Science,* 168(4), 521-530.

Cakmak, I. & Marschner, H. (1987). Mechanism of phosphorus‐induced zinc deficiency in cotton. III. Changes in physiological availability of zinc in plants Is mail. *Physiologia Plantarum*, 70(1), 13-20.

Cheng, Y., Zhou, W., El Sheery, N.I., Peters, C., Li, M., Wang, X. & Huang, J. (2011). Characterization of the Arabidopsis glycerophosphodiester phosphodiesterase (GDPD) family reveals a role of the plastid‐localized AtGDPD1 in maintaining cellular phosphate homeostasis under phosphate starvation. *Plant Journal*, 66(5), 781-795.

Childs, C.W. (1970). Potentiometric study of equilibriums in aqueous divalent metal orthophosphate solutions. *Inorganic Chemistry*, 9(11), 2465-2469.

Chiou, T.J., Aung, K., Lin, S.I., Wu, C.C., Chiang, S.F. & Su, C.L. (2006). Regulation of phosphate homeostasis by microRNA in *Arabidopsis*. *Plant Cell*, 18(2), 412-421.

Clarkson, D.T. & Scattergood, C.B. (1982). Growth and phosphate transport in barley and tomato plants during the development of, and recovery from, phosphate-stress.*Journal of Experimental Botany,*33(5), 865-875.

de Campos, M.C., Pearse, S.J., Oliveira, R.S. & Lambers, H. (2013). Downregulation of net phosphorus-uptake capacity is inversely related to leaf phosphorus-resorption proficiency in four species from a phosphorus-impoverished environment. *Annals of Botany,* 111(3), 445-454.

Delhaize, E. & Randall, P.J. (1995). Characterization of a phosphate-accumulator mutant of Arabidopsis thaliana. *Plant Physiology*, 107(1), 207-213.

Deng, M., Bian, H., Xie, Y., Kim, Y., Wang, W., Lin, E., Zeng, Z., Guo, F., Pan, J., Han, N., Wang, J., Qian, Q. & Zhu, M. (2011). Bcl-2 suppresses hydrogen peroxide-induced programmed cell death via *OsVPE2* and *OsVPE3*, but not via *OsVPE1* and *OsVPE4*, in rice. *FEBS Journal,* 278(24), 4797-4810.

Dong, B., Rengel, Z. & Delhaize, E. (1998). Uptake and translocation of phosphate by pho2 mutant and wild-type seedlings of *Arabidopsis thaliana.* *Planta*, 205(2), 251-256.

Fabre, D., Yin, X., Dingkuhn, M., Clément-Vidal, A., Roques, S., Rouan, L., Soutiras, A. & Luquet, D. (2019). Is triose phosphate utilization involved in the feedback inhibition of photosynthesis in rice under conditions of sink limitation? *Journal of Experimental Botany*, 70(20), 5773-5785.

Flóhe, L. & Ötting, F. (1984). Superoxide dismutase assays. In Methods in enzymology (eds L. Packer), Vol. 105, pp. 93-104, Academic Press, Orlando.

Flügge, U.I. (1992). Reaction mechanism and asymmetric orientation of the reconstituted chloroplast phosphate translocator. *Biochim Biophys Acta Biomembranes*, 1110(1), 112-118.

Flügge, U.I. & Heldt, H.W. (1984). The phosphate-triose phosphate-phosphoglycerate translocator of the chloroplast. *Trends in Biochemical Sciences,* 9(12), 530-533.

Franco-Zorrilla, J.M., Valli, A., Todesco, M., Mateos, I., Puga, M.I., Rubio-Somoza, I., Leyva, A., Weigel, D., García, J.A. & Paz-Ares, J. (2007). Target mimicry provides a new mechanism for regulation of microRNA activity. *Nature Genetics*, 39(8), 1033-1037.

Fujii, H., Chiou, T.J., Lin, S.I., Aung, K. & Zhu, J.K. (2005). A miRNA involved in phosphate-starvation response in *Arabidopsis*. *Current Biology*, 15(22), 2038-2043.

Fukayama, H., Ueguchi, C., Nishikawa, K., Katoh, N., Ishikawa, C., Masumoto, C., Hatanaka, T. & Misoo, S. (2012). Overexpression of Rubisco activase decreases the photosynthetic CO2 assimilation rate by reducing Rubisco content in rice leaves. *Plant Cell Physiology,* 53(6), 976-986.

Furihata, T., Suzuki, M. & Sakurai, H. (1992). Kinetic characterization of two phosphate uptake systems with different affinities in suspension-cultured *Catharanthus roseus* protoplasts. *Plant and Cell Physiology*, 33(8), 1151-1157.

Ganesan, V. & Thomas, G. (2001). Salicylic acid response in rice: influence of salicylic acid on H2O2 accumulation and oxidative stress. *Plant Science*, 160(6), 1095-1106.

Gu, M., Chen, A., Sun, S. & Xu, G. (2016). Complex regulation of plant phosphate transporters and the gap between molecular mechanisms and practical application: what is missing? *Molecular Plant*, 9(3), 396-416.

Hawkesford, M., Horst, W., Kichey, T., Lambers, H., Schjoerring, J., Møller, I.S. & White, P. (2012). Functions of macronutrients. In *Marschner's mineral nutrition of higher plants* (eds Marschner, P.), pp. 135-189. Academic Press, London.

Hawkins, H.J., Hettasch, H., Mesjasz-Przybylowicz, J., Przybylowicz, W. & Cramer, M.D. (2008). Phosphorus toxicity in the Proteaceae: a problem in post-agricultural lands. *Scientia Horticulturae*, 117(4), 357-365.

Hayes, P.E., Guilherme Pereira, C., Clode, P.L. & Lambers, H. (2019). Calcium‐enhanced phosphorus toxicity in calcifuge and soil‐indifferent Proteaceae along the Jurien Bay chronosequence. *New Phytologist*, 221(2), 764-777.

Heuer, S., Gaxiola, R., Schilling, R., Herrera‐Estrella, L., López‐Arredondo, D., Wissuwa, M., Delhaize, E. & Rouached, H. (2017). Improving phosphorus use efficiency: a complex trait with emerging opportunities. *Plant Journal,* 90(5), 868-885.

Ishimaru, Y., Suzuki, M., Kobayashi, T., Takahashi, M., Nakanishi, H., Mori, S. & Nishizawa, N.K. (2005). OsZIP4, a novel zinc-regulated zinc transporter in rice. *Journal of Experimental Botany*, 56(422), 3207-3214.

Kai, M., Masuda, Y., Kikuchi, Y., Osaki, M. & Tadano, T. (1997). Isolation and characterization of a cDNA from *Catharanthus roseus* which is highly homologous with phosphate transporter. *Soil Science and Plant Nutrition*, 43(1), 227-235.

Kirkby, E. (2012). Introduction, definition and classification of nutrients. In *Marschner's Mineral Nutrition of Higher Plants* (ed Marschner, P.) (pp. 3-5). Academic Press. London.

Klughammer, C. & Schreiber, U. (1994). An improved method, using saturating light pulses, for the determination of photosystem I quantum yield via P700+-absorbance changes at 830 nm. *Planta*, 192(2), 261-268.

Kramer, D.M., Johnson, G., Kiirats, O. & Edwards, G.E. (2004). New fluorescence parameters for the determination of QA redox state and excitation energy fluxes. *Photosynthesis Reserch,* 79(2), 209-218.

Krężel, A. & Maret, W. (2016). The biological inorganic chemistry of zinc ions. *Archives of Biochemistry and Biophysics*, 611, 3-19.

Krieger-Liszkay, A. (2005). Singlet oxygen production in photosynthesis. *Journal of Experimental Botany*, 56(411), 337-346.

Kurita, Y., Baba, K.I., Ohnishi, M., Anegawa, A., Shichijo, C., Kosuge, K., Fukaki, H. & Mimura, T. (2014). Establishment of a shortened annual cycle system; a tool for the analysis of annual re-translocation of phosphorus in the deciduous woody plant (*Populus alba* L.). *Journal of Plant Reserch*, 127(4), 545-551.

Lambers, H., Juniper, D., Cawthray, G.R., Veneklaas, E.J. & Martínez-Ferri, E. (2002). The pattern of carboxylate exudation in *Banksia grandis* (Proteaceae) is affected by the form of phosphate added to the soil. *Plant and Soil*, *238*(1), 111-122.

Lee, H.S., Lee, D.H., Cho, H.K., Kim, S.H., Auh, J.H. & Pai, H.S. (2015). InsP6-sensitive variants of the Gle1 mRNA export factor rescue growth and fertility defects of the *ipk1* low-phytic-acid mutation in *Arabidopsis*. *Plant Cell*, 27(2), 417-431.

Lee, R.B., Ratcliffe, R.G., & Southon, T.E. (1990). 31P NMR measurements of the cytoplasmic and vacuolar Pi content of mature maize roots: relationships with phosphorus status and phosphate fluxes. *Journal of Experimental Botany*, 41(9),: 1063-1078.

Leggewie, G., Willmitzer, L. & Riesmeier, J.W. (1997). Two cDNAs from potato are able to complement a phosphate uptake-deficient yeast mutant: identification of phosphate transporters from higher plants. *Plant Cell*, 9(3), 381-392.

Lemtiri-Chlieh, F., MacRobbie, E.A. & Brearley, C.A. (2000). Inositol hexakisphosphate is a physiological signal regulating the K+-inward rectifying conductance in guard cells. *Proceedings of the National Academy of Sciences,* 97(15), 8687-8692.

Lemtiri-Chlieh, F., MacRobbie, E.A., Webb, A.A., Manison, N.F., Brownlee, C., Skepper, J.N., Chen, J., Prestwich, G.D. & Brearley, C.A. (2003). Inositol hexakisphosphate mobilizes an endomembrane store of calcium in guard cells. *Proceedings of the National Academy of Sciences,* 100(17), 10091-10095.

Lin, S.I., Santi, C., Jobet, E., Lacut, E., El Kholti, N., Karlowski, W.M., Verdeil, J.L., Breitler, J.C., Périn, C., Ko, S.S., Guiderdoni, E., Chiou, T.J. & Guiderdoni, E. (2010). Complex regulation of two target genes encoding SPX-MFS proteins by rice miR827 in response to phosphate starvation. *Plant and Cell Physiology,* 51(12), 2119-2131.

Liu, C., Muchhal, U.S., Uthappa, M., Kononowicz, A.K. & Raghothama, K.G. (1998). Tomato phosphate transporter genes are differentially regulated in plant tissues by phosphorus. *Plant Physiology*, 116(1), 91-99.

Liu, F., Wang, Z., Ren, H., Shen, C., Li, Y., Ling, H. Q., Wu, C., Lian, X. & Wu, P. (2010). OsSPX1 suppresses the function of OsPHR2 in the regulation of expression of *OsPT2* and phosphate homeostasis in shoots of rice. *Plant Journal*, 62(3), 508-517.

Liu, J., Yang, L., Luan, M., Wang, Y., Zhang, C., Zhang, B., Shi, J., Zhao, F.G., Lan, W. & Luan, S. (2015). A vacuolar phosphate transporter essential for phosphate homeostasis in *Arabidopsis*. *Proceedings of the National Academy of Sciences*, 112(47), E6571-E6578.

Liu, T.Y., Huang, T.K., Yang, S.Y., Hong, Y.T., Huang, S.M., Wang, F.N., Chiang, S.F., Tsai, S. Y., Lu, W.C. & Chiou, T.J. (2016). Identification of plant vacuolar transporters mediating phosphate storage. *Nature Communications,* doi:10.1038/ncomms11095.

Loneragan, J.F., Grove, T.S., Robson, A.D. & Snowball, K. (1979). Phosphorus Toxicity as a Factor in Zinc-Phosphorus Interactions in Plants 1. *Soil Science Society of America Journal,* 43(5), 966-972.

Maenz, D.D., Engele-Schaan, C.M., Newkirk, R.W. & Classen, H.L. (1999). The effect of minerals and mineral chelators on the formation of phytase-resistant and phytase-susceptible forms of phytic acid in solution and in a slurry of canola meal. *Animal Feed Science and Technology,* 81(3-4), 177-192.

Makino, A., Mae, T. & Ohira, K. (1986). Colorimetric measurement of protein stained with Coomassie Brilliant Blue R on sodium dodecyl sulfate-polyacrylamide gel electrophoresis by eluting with formamide. *Agricultural and Biological Chemistry,* 50(7), 1911-1912.

Makino, A., Nakano, H. & Mae, T. (1994). Responses of ribulose-1, 5-bisphosphate carboxylase, cytochrome f, and sucrose synthesis enzymes in rice leaves to leaf nitrogen and their relationships to photosynthesis. *Plant Physiology*, 105(1), 173-179.

Makino, A., Nakano, H., Mae, T., Shimada, T. & Yamamoto, N. (2000). Photosynthesis, plant growth and N allocation in transgenic rice plants with decreased Rubisco under CO2 enrichment. *Journal of Experimental Botany*, 51(suppl\_1), 383-389.

Masumoto, C., Fukayama, H., Hatanaka, T. & Uchida, N. (2012). Photosynthetic characteristics of antisense transgenic rice expressing reduced levels of Rubisco activase. *Plant Production Science,* 15(3), 174-182.

Mekawy, A.M.M., Assaha, D.V., Munehiro, R., Kohnishi, E., Nagaoka, T., Ueda, A. & Saneoka, H. (2018). Characterization of type 3 metallothionein-like gene (OsMT-3a) from rice, revealed its ability to confer tolerance to salinity and heavy metal stresses. *Environmental and Experimental Botany*, 147, 157-166.

Mimura, T. (1995). Homeostasis and transport of inorganic phosphate in plants. *Plant and Cell Physiology*, 36(1), 1-7.

Mimura, T. (1999). Regulation of phosphate transport and homeostasis in plant cells. In *International Review of Cytology* (eds Kwang W. Jeon.), Vol. 191, pp. 149-200, Academic Press, New York.

Mimura, T., Dietz, K.J., Kaiser, W., Schramm, M.J., Kaiser, G. & Heber, U. (1990). Phosphate transport across biomembranes and cytosolic phosphate homeostasis in barley leaves. *Planta*, 180(2), 139-146.

Mimura, T., Sakano, K. & Shimmen, T. (1996). Studies on the distribution, re‐translocation and homeostasis of inorganic phosphate in barley leaves. *Plant, Cell & Environment*, 19(3), 311-320.

Mimura, T., Shindo, C., Kato, M., Yokota, E., Sakano, K., Ashihara, H. & Shimmen, T. (2000). Regulation of cytoplasmic pH under extreme acid conditions in suspension cultured cells of *Catharanthus roseus*: a possible role of inorganic phosphate. *Plant and Cell Physiology*, 41(4), 424-431.

Mimura, T., Yin, Z. H., Wirth, E. & Dietz, K.J. (1992). Phosphate transport and apoplastic phosphate homeostasis in barley leaves. *Plant and Cell Physiology*, 33(5), 563-568.

Mitsuhashi, N., Ohnishi, M., Sekiguchi, Y., Kwon, Y.U., Chang, Y.T., Chung, S.K., Inoue, Y., Reid, R.J., Yagisawa, H. & Mimura, T. (2005). Phytic acid synthesis and vacuolar accumulation in suspension-cultured cells of *Catharanthus roseus* induced by high concentration of inorganic phosphate and cations. *Plant Physiology*, 138(3), 1607-1614.

Miyagi, A., Takahashi, H., Takahara, K., Hirabayashi, T., Nishimura, Y., Tezuka, T., Kawai-Yamada, M. & Uchimiya, H. (2010). Principal component and hierarchical clustering analysis of metabolites in destructive weeds; polygonaceous plants. *Metabolomics,* 6(1), 146-155.

Miyagi, A., Noguchi, K., Tokida, T., Usui, Y., Nakamura, H., Sakai, H., Hasegawa, T. & Kawai-Yamada, M. (2019). Oxalate contents in leaves of two rice cultivars grown at a free-air CO2 enrichment (FACE) site. *Plant Production Science,* 22, 407-411.

Muchhal, U.S., Pardo, J.M. & Raghothama, K.G. (1996). Phosphate transporters from the higher plant *Arabidopsis thaliana*. *Proceedings of the National Academy of Sciences*, 93(19), 10519-10523.

Murphy, A.M., Otto, B., Brearley, C.A., Carr, J.P. & Hanke, D.E. (2008). A role for inositol hexakisphosphate in the maintenance of basal resistance to plant pathogens. *Plant Journal*, 56(4), 638-652.

Müller, P., Li, X.P. & Niyogi, K.K. (2001). Non-photochemical quenching. A response to excess light energy. *Plant Physiology*, 125(4), 1558-1566.

Nagy, R., Grob, H., Weder, B., Green, P., Klein, M., Frelet-Barrand, A., Schjoerring J.K., Brearley, C. & Martinoia, E. (2009). The Arabidopsis ATP-binding cassette protein AtMRP5/AtABCC5 is a high affinity inositol hexakisphosphate transporter involved in guard cell signaling and phytate storage. *Journal of Biological Chemistry,* 284(48), 33614-33622.

Nakamura, Y. (2013). Phosphate starvation and membrane lipid remodeling in seed plants. *Progress in Lipid Research,* 52(1), 43-50.

Nichols, D.G. & Beardsell, D.V. (1981). Interactions of calcium, nitrogen and potassium with phosphorus on the symptoms of toxicity in Grevillea cv.‘Poorinda Firebird’. *Plant and Soil*, 61(3), 437-445.

Noguchi, K., Tsunoda, T., Miyagi, A., Kawai-Yamada, M., Sugiura, D., Miyazawa, S. I., Tokida, T., Usui, Y., Nakamura, H., Sakai, H. & Hasegawa, T. (2018). Effects of elevated atmospheric CO2 on respiratory rates in mature leaves of two rice cultivars grown at a free-air CO2 enrichment site and analyses of the underlying mechanisms. *Plant and Cell Physiology,* 59(3), 637-649.

Nussaume, L., Kanno, S., Javot, H., Marin, E., Nakanishi, T.M. & Thibaud, M.C. (2011). Phosphate import in plants: focus on the PHT1 transporters. *Frontiers in Plant Science,* 2:83. doi: 10.3389/fpls.2011.00083.

Ogawa, S., Suzuki, Y., Yoshizawa, R., Kanno, K. & Makino, A. (2012). Effect of individual suppression of RBCS multigene family on Rubisco contents in rice leaves. *Plant, Cell & Environment,* 35(3), 546-553.

Ogo, Y., Itai, R.N., Nakanishi, H., Inoue, H., Kobayashi, T., Suzuki, M., Takahashi, M., Mori, S. & Nishizawa, N.K. (2006). Isolation and characterization of IRO2, a novel iron-regulated bHLH transcription factor in graminaceous plants. *Journal of Experimental Botany,* 57(11), 2867-2878.

Ova, E.A., Kutman, U.B., Ozturk,L. & Cakmak, I. (2015). High phosphorus supply reduced zinc concentration of wheat in native soil but not in autoclaved soil or nutrient solution. *Plant and Soil,* 393(1-2), 147-162.

Parks, S.E., Haigh, A.M. & Cresswell, G.C. (2000). Stem tissue phosphorus as an index of the phosphorus status of *Banksia ericifolia* L. f. *Plant and Soil*, 227(1-2), 59-65.

Perera, I., Fukushima, A., Arai, M., Yamada, K., Nagasaka, S., Seneweera, S. & Hirotsu, N. (2019). Identification of low phytic acid and high Zn bioavailable rice (*Oryza sativa* L.) from 69 accessions of the world rice core collection. *Journal of Cereal Science,* 85, 206-213.

Perera, I., Seneweera, S., & Hirotsu, N. (2018). Manipulating the Phytic Acid Content of Rice Grain Toward Improving Micronutrient Bioavailability. *Rice*, 11(1), 4.

Pierre, W.H. & Parker, F.W. (1927). Soil phosphorus studies: II. The concentration of organic and inorganic phosphorus in the soil solution and soil extracts and the availibility of the organic phosphorus to plants. *Soil Science*, 24(2), 119-128.

Porra, R.J., Thompson, W.A. & Kriedemann, P.E. (1989). Determination of accurate extinction coefficients and simultaneous equations for assaying chlorophylls *a* and *b* extracted with four different solvents: verification of the concentration of chlorophyll standards by atomic absorption spectroscopy. *Biochimica et Biophysica Acta-Bioenergetics,* 975(3), 384-394.

Portis, A.R. (2003). Rubisco activase–Rubisco's catalytic chaperone. *Photosynthesis Research,* 75(1), 11-27.

Pospíšil, P. (2016). Production of reactive oxygen species by photosystem II as a response to light and temperature stress. *Frontiers in Plant Science,* 7:1950. doi: 10.3389/fpls.2016.01950.

Pratt, J., Boisson, A.M., Gout, E., Bligny, R., Douce, R. & Aubert, S. (2009). Phosphate (Pi) starvation effect on the cytosolic Pi concentration and Pi exchanges across the tonoplast in plant cells: an in vivo 31P-nuclear magnetic resonance study using methylphosphonate as a Pi analog. *Plant Physiology,* 151(3), 1646-1657.

Puga, M.I., Mateos, I., Charukesi, R., Wang, Z., Franco-Zorrilla, J.M., de Lorenzo, L., Irigoyen, M.L., Masiero, S., Bustos, R., Rodriguez, J., Leyva, A., Rubio, V., Sommer, H. & Leyva, A. (2014). SPX1 is a phosphate-dependent inhibitor of Phosphate Starvation Response 1 in *Arabidopsis*. *Proceedings of the National Academy of Sciences*, 111(41), 14947-14952.

Raboy, V., Gerbasi, P.F., Young, K.A., Stoneberg, S.D., Pickett, S.G., Bauman, A.T., Murthy, P.P., Sheridan, W.F. & Ertl, D.S. (2000). Origin and seed phenotype of maize low phytic acid 1-1 and low phytic acid 2-1. *Plant Physiology,* 124(1), 355-368.

Raghothama, K.G. (1999). Phosphate acquisition. *Annual Review of Plant Biology*, 50, 665-693.

Ranieri, A., Castagna, A., Baldan, B. & Soldatini, G.F. (2001). Iron deficiency differently affects peroxidase isoforms in sunflower. *Journal of Experimental Botany*, 52(354), 25-35.

Rossiter, R.C. (1952). Phosphorus toxicity in subterranean clover and oats grown on Muchea sand, and the modifying effects of lime and nitrate-nitrogen. *Australian Journal of Agricultural Research,* 3(3), 227-243.

Rubio, V., Linhares, F., Solano, R., Martín, A.C., Iglesias, J., Leyva, A. & Paz-Ares, J. (2001). A conserved MYB transcription factor involved in phosphate starvation signaling both in vascular plants and in unicellular algae. *Genes and Development,* 15(16), 2122-2133.

Rokka, A., Zhang, L. & Aro, E.M. (2001). Rubisco activase: an enzyme with a temperature‐dependent dual function?. *Plant Journal,* 25(4), 463-471.

Sacksteder, C.A. & Kramer, D.M. (2000). Dark-interval relaxation kinetics (DIRK) of absorbance changes as a quantitative probe of steady-state electron transfer. *Photosynthesis Research*, 66(1-2), 145-158.

Sakano, K. (1990). Proton/phosphate stoichiometry in uptake of inorganic phosphate by cultured cells of *Catharanthus roseus* (L.) G. Don. *Plant Physiology,* 93(2), 479-483.

Salvucci, M.E., Portis, A.R. & Ogren, W.L. (1985). A soluble chloroplast protein catalyzes ribulosebisphosphate carboxylase/oxygenase activation in vivo. *Photosynthesis Research,* 7(2), 193-201.

Secco, D., Wang, C., Arpat, B.A., Wang, Z., Poirier, Y., Tyerman, S.D., Wu, P., Shou, H. & Whelan, J. (2012). The emerging importance of the SPX domain‐containing proteins in phosphate homeostasis. *New Phytologist,* 193(4), 842-851.

Shane, M.W., McCully, M.E. & Lambers, H. (2004a). Tissue and cellular phosphorus storage during development of phosphorus toxicity in *Hakea prostrata* (Proteaceae). *Journal of Experimental Botany*, 55(399), 1033-1044.

Shane, M.W., Szota, C. & Lambers, H. (2004b). A root trait accounting for the extreme phosphorus sensitivity of *Hakea prostrata* (Proteaceae). *Plant, Cell & Environment,* 27(8), 991-1004.

Shane, M.W. & Lambers, H. (2006). Systemic suppression of cluster-root formation and net P-uptake rates in *Grevillea crithmifolia* at elevated P supply: a proteacean with resistance for developing symptoms of ‘P toxicity’. *Journal of Experimental Botany*, 57(2), 413-423.

Sharkey, T.D. (1985). Photosynthesis in intact leaves of C3 plants: physics, physiology and rate limitations. *Botanical Review*, 51(1), 53-105.

Shive, J.W. (1918). Toxicity of monobasic phosphates towards soybeans grown in soil-and solution-cultures. *Soil Science*, 5, 87-122.

Singh, J.P., Karamanos, R.E. & Stewart, J.W.B. (1988). The mechanism of phosphorus-induced zinc deficiency in bean (*Phaseolus vulgaris* L.). *Canadian Journal of Soil Science,* 68(2), 345-358.

Skene, K.R. (1998). Cluster roots: some ecological considerations. *Journal of Ecology*, 86(6), 1060-1064.

Sonoike, K. (2011). Photoinhibition of photosystem I. *Physiologia Plantarum*, 142(1), 56-64.

Specht, R.L. (1963). Dark Island heath (Ninety-Mile Plain, South Australia). VII. The effect of fertilizers on composition and growth, 1950-60. *Australian Journal of Botany*, 11(1), 67-94.

Su, D., Zhou, L., Zhao, Q., Pan, G. & Cheng, F. (2018). Different phosphorus supplies altered the accumulations and quantitative distributions of phytic acid, zinc, and iron in rice (*Oryza sativa* L.) Grains. *Journal of Agricultural and Food Chemistry,* 66(7), 1601-1611.

Suganami, M., Suzuki, Y., Sato, T. & Makino, A. (2018). Relationship between Rubisco activase and Rubisco contents in transgenic rice plants with overproduced or decreased Rubisco content. *Soil Science and Plant Nutrition,* 64(3), 352-359.

Suzuki, M., Tanaka, K., Kuwano, M. & Yoshida, K.T. (2007). Expression pattern of inositol phosphate-related enzymes in rice (*Oryza sativa* L.): implications for the phytic acid biosynthetic pathway. *Gene*, 405(1-2), 55-64.

Suzuki, Y., Fujimori, T., Kanno, K., Sasaki, A., Ohashi, Y. & Makino, A. (2012). Metabolome analysis of photosynthesis and the related primary metabolites in the leaves of transgenic rice plants with increased or decreased Rubisco content. *Plant, Cell & Environment,* 35(8), 1369-1379.

Suzuki Y, Kawazu T. & Koyama, H. (2004). RNA isolation from siliques, dry seeds and other tissues of *Arabidopsis thaliana*. *Biotechniques,* 37(4), 542-544.

Suzuki, Y., Ohkubo, M., Hatakeyama, H., Ohashi, K., Yoshizawa, R., Kojima, S., Hayakawa, T., Yamaya, T., Mae, T. & Makino, A. (2007). Increased Rubisco content in transgenic rice transformed with the ‘sense’rbcS gene. *Plant and Cell Physiology,* 48(4), 626-637.

Stevenson-Paulik, J., Bastidas, R.J., Chiou, S.T., Frye, R.A. & York, J.D. (2005). Generation of phytate-free seeds in *Arabidopsis* through disruption of inositol polyphosphate kinases. *Proceedings of the National Academy of Sciences,* 102(35), 12612-12617.

Takagi, D., Amako, K., Hashiguchi, M., Fukaki, H., Ishizaki, K., Goh, T., Fukao, Y., Sano, R., Kurata, T., Demura, T., Sawa, S. & Miyake, C. (2017). Chloroplastic ATP synthase builds up a proton motive force preventing production of reactive oxygen species in photosystem I. *Plant Journal,* 91(2), 306-324.

Takagi, D., Hashiguchi, M., Sejima, T., Makino, A. & Miyake, C. (2016b). Photorespiration provides the chance of cyclic electron flow to operate for the redox-regulation of P700 in photosynthetic electron transport system of sunflower leaves. *Photosynthesis Research,* 129(3), 279-290.

Takagi, D., Ihara, H., Takumi, S. & Miyake, C. (2019). Growth light environment changes the sensitivity of photosystem I photoinhibition depending on common wheat cultivars. *Frontiers in Plant Science,* 10:686. doi: 10.3389/fpls.2019.00686.

Takagi, D., Takumi, S., Hashiguchi, M., Sejima, T. & Miyake, C. (2016a). Superoxide and singlet oxygen produced within the thylakoid membranes both cause photosystem I photoinhibition. *Plant Physiology*, 171(3), 1626-1634.

Terashima, I., Funayama, S. & Sonoike, K. (1994). The site of photoinhibition in leaves of Cucumis sativus L. at low temperatures is photosystem I, not photosystem II. *Planta,* 193(2), 300-306.

Ullrich-Eberius, C.I., Novacky, A., Fischer, E. & Lüttge, U. (1981). Relationship between energy-dependent phosphate uptake and the electrical membrane potential in *Lemna gibba* G1. *Plant Physiology,* 67(4), 797-801.

Ullrich-Eberius, C.I., Novacky, A. & Van Bel, A.J.E. (1984). Phosphate uptake in *Lemna gibba* G1: energetics and kinetics. *Planta*, *161*(1), 46-52.

Vargas-Suárez, M., Ayala-Ochoa, A., Lozano-Franco, J., García-Torres, I., Díaz-Quiñonez, A., Ortíz-Navarrete, V.F. & Sánchez-de-Jiménez, E. (2004). Rubisco activase chaperone activity is regulated by a post-translational mechanism in maize leaves. *Journal of Experimental Botany,* 55(408), 2533-2539.

von Caemmerer, S.V., & Farquhar, G.D. (1981). Some relationships between the biochemistry of photosynthesis and the gas exchange of leaves. *Planta*, 153(4), 376-387.

Wada, S., Suzuki, Y., Takagi, D., Miyake, C. & Makino, A. (2018). Effects of genetic manipulation of the activity of photorespiration on the redox state of photosystem I and its robustness against excess light stress under CO2-limited conditions in rice. *Photosynthesis Research*, 137(3), 431-441.

Wang, C., Ying, S., Huang, H., Li, K., Wu, P. & Shou, H. (2009). Involvement of *OsSPX1* in phosphate homeostasis in rice. *Plant Journal*, 57(5), 895-904.

Wang, Z., Ruan, W., Shi, J., Zhang, L., Xiang, D., Yang, C., Li, C., Wu, Z., Liu, Y., Shou, H., Mo, X., Mao, C. & Shou, H. (2014). Rice SPX1 and SPX2 inhibit phosphate starvation responses through interacting with PHR2 in a phosphate-dependent manner.  *Proceedings of the National Academy of Sciences*, 111(41), 14953-14958.

White, P.J. (2012). Ion uptake mechanisms of individual cells and roots: Short-disgance transport. In *Marschner's Mineral Nutrition of Higher Plants* (ed Marschner, P.) (pp. 7-47). Academic Press. London.

Yamori, W., Masumoto, C., Fukayama, H. & Makino, A. (2012). Rubisco activase is a key regulator of non‐steady‐state photosynthesis at any leaf temperature and, to a lesser extent, of steady‐state photosynthesis at high temperature. *Plant Journal*, 71(6), 871-880.

Yuan, M., Li, X., Xiao, J. & Wang, S. (2011). Molecular and functional analyses of COPT/Ctr-type copper transporter-like gene family in rice. *BMC Plant Biology*, 11: 69. doi: 10.1186/1471-2229-11-69.

Zhang, N., Kallis, R.P., Ewy, R.G. & Portis, A.R. (2002). Light modulation of Rubisco in Arabidopsis requires a capacity for redox regulation of the larger Rubisco activase isoform. *Proceedings of the National Academy of Sciences*, 99(5), 3330-3334.

Zhou, J., Jiao, F., Wu, Z., Li, Y., Wang, X., He, X., Zhong, W. & Wu, P. (2008). OsPHR2 is involved in phosphate-starvation signaling and excessive phosphate accumulation in shoots of plants. *Plant Physiology*, 146(4), 1673-1686.

Zhu, Y.G., Smith, S.E. & Smith, F.A. (2001). Zinc (Zn)-phosphorus (P) interactions in two cultivars of spring wheat (*Triticum aestivum* L.) differing in P uptake efficiency. *Annals of Botany*, *88*(5), 941-945.

**Figure legends**

**Figure 1**

Growth phenotypes of rice plants grown under different inorganic phosphate (Pi) application rates. (a) Representative plants grown under different Pi application rates on the 70th day after germination. White bars indicate a scale of 10 cm. At this stage, the plant height (b) and chlorophyll content in the leaf blades (c) were examined. (d) Dry matter of the leaf blade, leaf sheath, and root in rice plants grown under different Pi application rates. Results are expressed as means ± SD (n = 6-8). Different letters indicate significant differences between different Pi application rates (Tukey’s HSD test, *p* < 0.05).

**Figure 2**

CO2 assimilation (*A*) and photosynthetic parameters assessed by using chlorophyll fluorescence to the change in the internal CO2 concentration (Ci) in rice leaf blades (a) Results of *A* in rice plants grown under 0.06 and 0.6 mM Pi, and (e) *A* in rice plants grown under 0.6 to 3.0 mM Pi. The results of the 0.6 mM Pitreatmentwere the same in (a) and (e). (b), (c), and (d) Y(II), Y(NPQ), and Y(NO), respectively, in rice plants grown under 0.06 and 0.6 mM Pi. (f), (g), and (h) Results of Y(II), Y(NPQ), and Y(NO) in rice plants grown under 0.6 to 3.0 mM Pi. The results of the 0.6 mM Pitreatmentwere the same in (b) and (f), (c) and (g), and (d) and (h). (i) Initial slope of *A* toward the increase in Ci under low Ci conditions. (j) shows the *F*v/*F*m in rice leaf blades grown under different Pi application conditions. Results are expressed as means ± SD (n = 4-6). Different letters in (i) and (j) indicate significant differences among different Pi conditions (Tukey’s HSD test, *p* < 0.05).

**Figure 3**

Effects of Pi application on the sugar phosphate and adenylate concentrations in rice leaf blades. (a) Scheme of sugar phosphate metabolism in chloroplasts and cytosol. The sugar phosphate concentrations represented by red characters indicates those quantified in this study. (b) Sugar phosphate concentration in rice leaf blades. White, blue, dark blue, purple, pink, and red bars indicate low (0.06 mM), control (0.6 mM), 1.2 mM, 1.8 mM, 2.4 mM, and 3.0 mM treatment conditions, respectively. (c) and (d) Adenylate concentration and ATP/ADP ratio in rice leaf blades. Results are expressed as means ± SD (n = 3-9). Different letters indicate significant differences among different Pi conditions (Tukey’s HSD test, *p* < 0.05).

**Figure 4**

Effects of P toxicity on Rubisco concentration and activation by Rubisco activase (RCA). (a) Leaf nitrogen content in rice leaf blades and (b) Rubisco concentration in rice leaf blades (n = 3-4). The Rubisco activation was calculated from the ratio of the initial to maximum Rubisco activity (c), and the carbamylation potential was calculated from the ratio of the total to the maximum Rubisco activity (d) (n = 3-5). The white and dark grey bars indicate the results of the leaves sampled under illumination and at night, respectively, under control-Pi conditions. (e) Result of the western blot analysis, which targets RCA in rice leaves. Red arrows and Greek characters indicate the isoforms of RCA. Each sample was loaded on a leaf area basis (0.02 cm2). (f) and (g) Relative concentrations of the total RCA and each isoform evaluated from the western blot analysis. The RCA concentration in the control-Pi plants is set as “1” and the relative content is shown. The black, red, and grey characters above the bars indicate the statistical results for the *α,* *β,* and *β\** isoforms, respectively. (h) and (i) Isoform ratio between *α*/*β* and *β*\*/*β* (n = 4). Results are expressed as means ± SD. Different letters indicate significant differences among different Pi conditions (Tukey’s HSD test, *p* < 0.05).

**Figure 5**

Ascorbate peroxidase (APX) and superoxide dismutase (SOD) activities in rice leaf blades grown under different Pi conditions. (a) Results of the total, cytosolic, and chloroplastic APX activities evaluated on a leaf area basis. The red, yellow, and green characters above the bars indicate the statistical results of the total, cytosolic, and chloroplastic APX activities, respectively. (b) Results of the chloroplastic APX activities evaluated on a chlorophyll basis. (c) Results of the total, Fe-, Mn-, and Cu/Zn-SOD activities evaluated on a leaf area basis. The grey and blue characters above the bars indicate the statistical results of the total and Cu/Zn-SOD activities, respectively. Results are expressed as means ± SD (n = 3). Different letters indicate significant differences among different Pi conditions (Tukey’s HSD test, *p* < 0.05).

**Figure 6**

mRNA expressions of Cu/Zn-SOD genes in rice leaf blades. The mRNA expression is expressed on a fresh weight (F.W.) (red bars) and 18S ribosome expression (blue bars) basis. Results are expressed as means ± SD (n = 3-4). Different letters indicate significant differences among different Pi conditions (Tukey’s HSD test, *p* < 0.05).

**Figure 7**

Leaf mineral concentration and metal-responsive gene expression in rice leaf blades grown under different Pi conditions. The concentration of each mineral nutrient was quantified on the leaf dry matter basis (a). Results are expressed as means ± SD (n = 3-4). (b) mRNA expressions of the metal-deficiency-responsive genes *ZIP4*, *IRO2*, and *COPT1* in the leaves. The mRNA expression is expressed on a fresh weight (F.W.) (red bars) and 18S ribosome expression (blue bars) basis. Results are expressed as means ± SD (n = 3-7). Different letters indicate significant differences among different Pi conditions (Tukey’s HSD test, *p* < 0.05).

**Figure 8**

mRNA expression involved in phytic acid synthesis and phytic acid concentration in rice leaf blades. (a) Scheme of phytic acid synthesis in plants (Penera et al., 2019). (b) mRNA expression involved in phytic acid synthesis is expressed on a fresh weight (F.W.) (red bars) and 18S ribosome expression (blue bars) basis. (c) and (d) Leaf phytic acid concentration expressed on a F.W. and leaf area basis, respectively. (e) Molecular ratio of phytic acid to free Pi in leaves. Results are expressed as mean ± SD (n = 3-4). Different letters indicate significant differences among different Pi conditions (Tukey’s HSD test, *p* < 0.05).

**Figure 9**

Scheme of P toxicity in plant cells. Under normal P concentration (a), phytic acid synthesis is maintained to produce its proper concentration in the cytosol, preventing any negative effects of phytic acid on physiological functions within the plant cell. Under excessive Pi accumulation (b), an increase in the cytosolic sugar phosphate concentration activates phytic acid synthesis, and an increase in the phytic acid concentration causes Zn precipitation, which, in turn, decreases Cu/Zn-SOD activities. Simultaneously, RCA concentration decreases depending on the excessive accumulation of Pi, and photosynthesis is limited by the decrease in electron sink activities, because of the decrease in Rubisco activation. The decrease in electron sink activities leads to a decrease in ATP and NADPH consumptions. Subsequently, electrons accumulate in the PET chain, stimulating reactive oxygen species (ROS) production. ROS accumulation is further intensified by a decrease in Cu/Zn-SOD activities. As a result, higher ROS accumulation triggers leaf necrosis in land plants.

**Table 1**

Pi and Po concentrations in rice leaf blades. The different letters indicate significant differences between each Pi application (Tukey’s HSD test, *p* < 0.05).