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Research Article

The Expression of *OsPLA2-III* and *OsPPO* Genes in Rice (*Oryza sativa* L.) Under Fe Toxicity Stress

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ABSTRACT

Lipids are an important biomolecule in plants because of their structural and functional roles in plant cells. Moreover, they could act as signal molecules in the defense system of plants suffering from biotic and abiotic stresses. Furthermore, plants develop various tolerance strategies to cope with iron (Fe) toxicity, for example, by involving genes in the detoxification process and other mechanisms. Therefore, the objective of this research was to investigate the expression of *OsPLA2-III* and *OsPPO* genes during Fe stress conditions. It was carried out using two-week-old seedlings of two rice varieties, namely, IR64 (Fe-sensitive variety) and Pokkali (Fe-tolerant variety). The seedlings were treated with 400 ppm $FeSO_4.7H_2O$ in the nutrient culture solution and compared with control that received 1 ppm $FeSO_4.7H_2O$. Furthermore, leaf bronzing, chlorophyll content and relative expression of *OsPLA2-III* and *OsPPO* genes were observed. An in-silico study was also performed to predict the interaction between OsPLA2-III and OsPPO proteins. The results showed that the Fe toxicity induced leaf bronzing, decreased leaf chlorophyll content, and increased the expression levels of *OsPLA2-III* and *OsPPO* genes. Therefore, both genes were suggested to have a role in plant tolerance mechanism during Fe toxicity stress through the lipid signaling pathway.

Keywords: Fe toxicity, In-silico analysis, Lipid, Signal molecules

Introduction

As an essential micronutrient, Fe is required by plants in small amounts to carry out various reactions in cell metabolisms. The Fe element also acts as a co-factor of various enzymes that play important roles in metabolic processes, such as photosynthesis and respiration [1]. However, this element is toxic for plants at high concentrations in the cell or the growth medium. As an active-redox element, Fe is able to cause oxidative stress in cells through Haber-Weiss and Fenton reactions. This stress could then damage genetic materials, proteins, and cell membranes [2].

Cell membranes play important roles in plant cells both structurally and functionally. Additionally, lipid as a building block of these membranes has an important function in the growth, development, and response of plants to biotic and abiotic stresses. The lipid-signaling process is known as a mechanism of lipid biosynthesis in cells [3, 4]. According to Wang & Chapman [5], it plays a role in various cellular and physiological processes. Lipids are known to be involved in plant responses to hormones (abscisic acid and auxin), abiotic stresses, plant-microbial interactions, and plant growth and development.

Lipid signaling involves various enzymes, na-

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mely phospholipase D (PLD), phospholipase A (PLA), acyl hydrolases, phytosphingosine kinases, diacylglycerol kinases, and fatty acid amide hydrolases [5]. However, to date, its activity in plants under Fe toxicity stress conditions has never been studied.

Plants exposed to Fe toxicity show symptoms like brown spots on their leaf blade or bronzing. According to Peng & Yamauchi [6], bronzing occurs due to the deposition of melanin compounds and chlorophyll degradation associated with the senescence process. The brown pigment leading to bronzing on leaves is a phenolic compound oxidized by polyphenol oxidase (PPO) enzyme [7].

Bronzing in rice leaf is a polygenic trait and is controlled by various loci on several chromosomes [8]. One of the genes that is expected to involve controlling bronzing trait is OsPPO gene, which encodes polyphenol oxidase enzyme responsible for the oxidation process of phenolic compounds to produce melanin. In addition, the PPO enzyme is also reported as an antioxidative enzyme that is produced in plants under stress conditions [9, 10].

Another gene that might contribute to Fe tolerance in plants is *OsPLA2-III* gene. The gene encodes phospholipase A2 involved in the lipid signaling process [3, 11]. Our previous study showed that linoleic and linolenic acid were metabolite markers related to rice response to Fe toxicity [12]. In this study, the expression of *OsPLA2-III* and *OsPPO* genes at different Fe concentrations was investigated. The expression data of *OsPLA2-III* and *OsPPO* genes would be useful as basic data for utilizing the genes in the development of new rice lines, tolerant to Fe toxicity.

Material and Methods

Experimental conditions

The experiment was a factorial experiment and was arranged in a complete randomized design with three replications. Moreover, the first factor consisted of one Fe treatment (400 ppm $FeSO_4.7H_2O$ (Fe-toxicity) and control (1 ppm). The second factor was two rice varieties, i.e., IR64 and Pokkali.

Two-week-old rice seedlings were exposed to Fe-treatment in half-strength nutrient culture solutions [13] supplemented with 0.2% (w/v) agar powder [14, 15] for five days. Leaf bronzing score was then recorded 5 days after treatment (DAT), while leaf chlorophyll and gene expression analyses were conducted at 0, 1, 3, and 5 DAT.

Analysis of chlorophyll content

Chlorophyll was extracted from leaves at 0, 1, 3, and 5 DAT using acetone. Briefly, 0.1 g of fresh leaves were ground in a mortar containing 80% cold acetone. Then, the suspension was filtered using a Whatman paper. The extract was then centrifuged at 3000 rpm and at a temperature of 4 °C for 15 minutes. The absorbance of the supernatant was observed in a visible spectrophotometer (Thermo Spectronic GENESYS 20 Visible Spectrophotometer, Thermofisher Scientific Inc., USA) at 646 and 630 nm. Finally, the quantification of leaf chlorophyll content followed the formula used by Lichtenthaler [16].

Determination of leaf bronzing score

Leaf bronzing was observed and scored at 5 DAT according to Shimizu et al. [17] with minor modification. The bronzing was observed following the order of 2^{nd}, 3^{rd}, and 4^{th} leaf (15) and categorized into without symptom (N), bronzing only in leaf tip (T), 50% area of leaf showed bronzing (P), 100% area of leaf showed bronzing (W), and rolled (dead) leaf (R). The combination of these five categories was used for scoring that ranged from 1 to 10.

RNA isolation and cDNA synthesis

Total RNA was isolated from the leaves using TRIzolTM reagent (Invitrogen, USA) following the manufacturer's instructions. Furthermore, the cDNA synthesis was carried out using RevertAid First Strand cDNA Synthesis Kit (Thermofisher Scientific Inc., USA), also following the manufacturer's instructions. The cDNA was then stored at -80 ˚C for further analysis.

Gene expression analysis using quantitative real time-polymerase chain reaction (qRT-PCR)

The expression analyses of Actin, OsPLA2-III, and OsPPO genes were carried out using qRT-PCR, which was performed in triplicate using gene-specific primers. The Actin primers used were 5'-GGTTCATCAAGAAGGGACCCG-3' (forward) and 5'-TGGAGGTG-TATCCTGATGCGACAA-3' (reverse) [18]. Furthermore, the OsPLA2-III primers were 5'-CCAGGCCAAGAATGACTACCT-3' (forward) and 5'-GTCGATCATGCACTTGTTCCC-3' (reverse), while the PPO primers were 5'-

Table 1. Change in leaf chlorophyll content of rice var. IR64 and Pokkali due to the two Fe concentrations

Time period (day)	Varieties	Total leaf chlorophyll content (mg/g FW)	
		1 ppm	400 ppm
0	IR64	250.1 ± 14.9[a]	246.0 ± 12.5[a]
	Pokkali	263.3 ± 19.9[a]	247.9 ± 5.9[a]
1	IR64	233.9 ± 17.5[a]	235.3 ± 28.5[a]
	Pokkali	205.1 ± 26.6[a]	195.9 ± 13.3[a]
3	IR64	366.6 ± 13.5[d]	205.1 ± 17.9[a]
	Pokkali	311.7 ± 4.9[c]	218.5 ± 4.8[a]
5	IR64	360.3 ± 8.5[b]	250.9 ± 17.8[a]
	Pokkali	428.6 ± 18.2[c]	354.5 ± 11.3[b]

*)1 ppm = control and 400 ppm = Fe-toxicity conditions. [a-d]superscript letters following the numbers in each time period indicate significant differences based on Duncan's multiple range test (α=0.05). Data is shown as mean ± error standard.

GCGAGGACATGGGCATCT-3' (forward) and 5'-AGTCGGGGTCGGTGAAGTC-3' (reverse) [19].

The qRT-PCR reactions were prepared using DyNAmo Flash SYBR Green qPCR Kit (Thermofisher Scientific Inc., USA) following the manufacturer's instructions. Furthermore, the qRT-PCR program used is as follows, 95°C for 7 min, 40 cycles of denaturation at 95°C for 10 sec, annealing at 55°C for 10 sec (Actin), or 63°C for 10 sec (OsPLA2-III), or 48°C for 10 sec (OsPPO), and extension at 72°C for 20 sec. Finally, the relative expression of each gene was quantified based on the $2^{-\Delta\Delta Ct}$ formula (20).

In-silico analysis of the interaction between OsPLA2-III and OsPPO protein

Protein-protein interaction (PPI) and gene ontology analyses were performed using STRING ver. 11.0 software (https://string-db.org/) to predict the interaction between the proteins studied in this research and other proteins in the database.

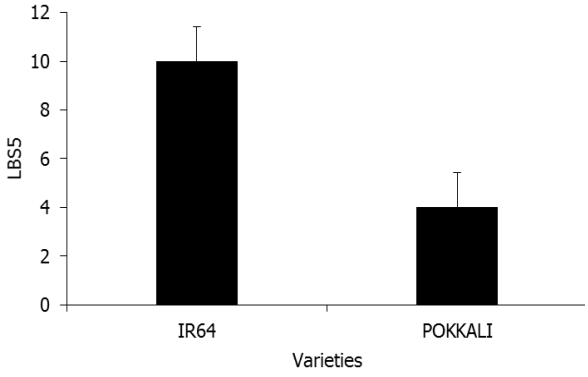


Figure 1. Leaf bronzing score of rice at 5 DAT. The bar represents error standard

Furthermore, protein databases for rice (*Oryza sativa* L.) in NCBI with taxonomy id 4530 were used in this *in-silico* analysis as a source of the protein sequence.

Data analysis

Two-way analysis of variance (ANOVA) at a significant level (α) of 5% was performed using SPSS 16.0 (SPSS Inc., Chicago, IL, USA).

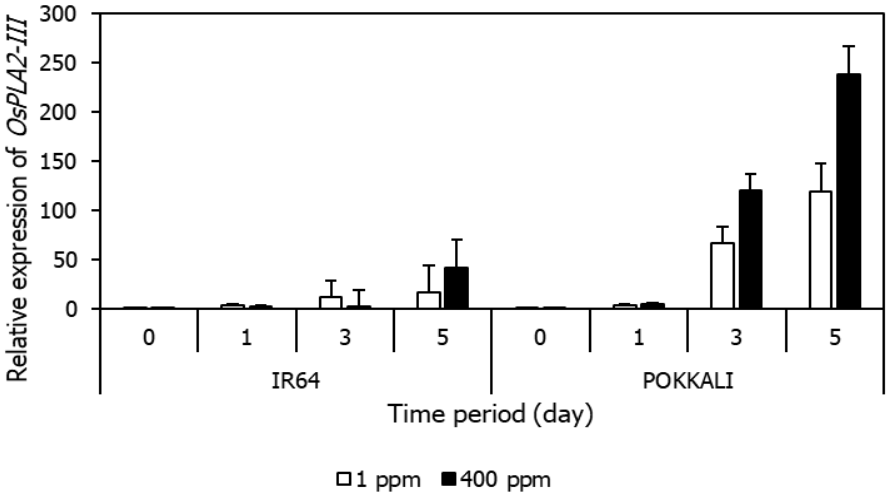
Results and Discussions

Leaf chlorophyll content and leaf bronzing score of two rice varieties in two Fe concentrations

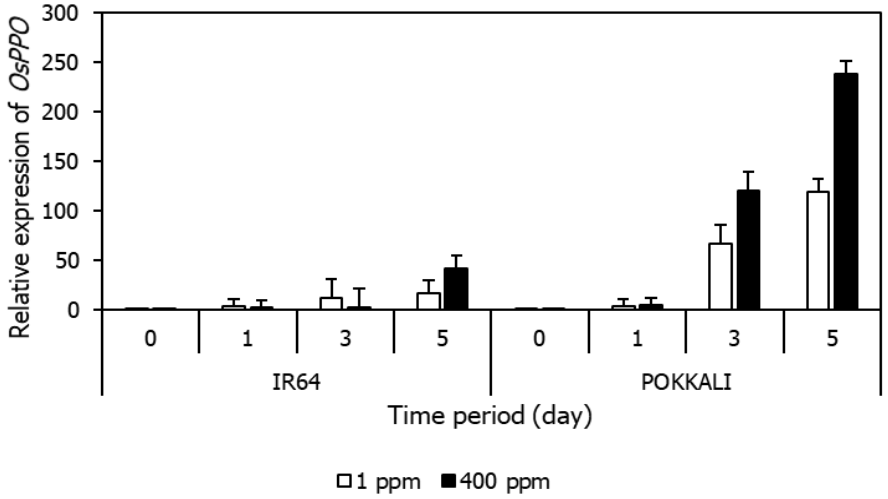
The concentration of Fe in the nutrition culture media significantly affected ($p < 0.05$) the leaf chlorophyll content of both rice varieties. Therefore, the chlorophyll content of both varieties decreased under 400 ppm Fe (toxic condition) until 5 DAT. The leaf chlorophyll content of rice var. Pokkali decreased more than that of rice var. IR64 due to the Fe toxicity treatments (Table 1). Furthermore, rice var. IR64 showed a higher leaf bronzing level (score = 10) compared to the rice var. Pokkali (score = 4) when both varieties were stressed with Fe toxicity (Figure 1).

The OsPLA2-III and OsPPO expression in two rice varieties under two Fe concentrations

The qRT-PCR result showed that the expression of *OsPLA2-III* and *OsPPO* genes increased in both varieties with the Fe toxicity treatment. A drastic increase in gene expression of both genes was shown by rice var. Pokkali (an Fe-tolerant variety) under Fe-toxicity condition (Figure 2a-b). In addition, the expression level of both genes in rice var. Pokkali was higher than that of rice var. IR64.



(a)



(b)

Figure 2. OsPLA2-III (a) and OsPPO (b) gene expression under two Fe concentrations. The bar represents ± standard error

In-silico analysis between OsPLA2-III and OsPPO protein

A prediction network associated with OsPLA2-III and OsPPO proteins was constructed based on a web-based public database. The *in-silico* analysis showed that various proteins associated with OsPLA2-III and OsPPO proteins were grouped into two clusters (Figure 3). Furthermore, both proteins were grouped in a different cluster protein network, and Cluster 1 (network of OsPLA2-III) and cluster 2 (network of OsPPO) were connected to OS07T0191000-01 and OS02T0709200-01, respectively. This study also showed that among all identified proteins that were related to both proteins, 43 were involved in biological process, 19 in molecular process, and 2 in cellular component. Those protein categories were identified with 82 genes based on gene ontology (GO) enrichment analysis of OsPLA2-III and OsPPO proteins (Figure 4). In biological process aspect, phospholipid metabolic process (GO:0006644), organophosphate metabolic process (GO:0019637), primary metabolic process (GO:0044238), cellular metabolic process (GO:0044237), organic substance metabolic process (GO:0071704), phosphate-containing compound metabolic process (GO:0006796), and organic substance catabolic process (GO:1901575) were identified for OsPLA2-III and OsPPO proteins associated network. In the molecular process aspect, catalytic activity (GO:0003824) and ion binding (GO:0043167) were identified for these

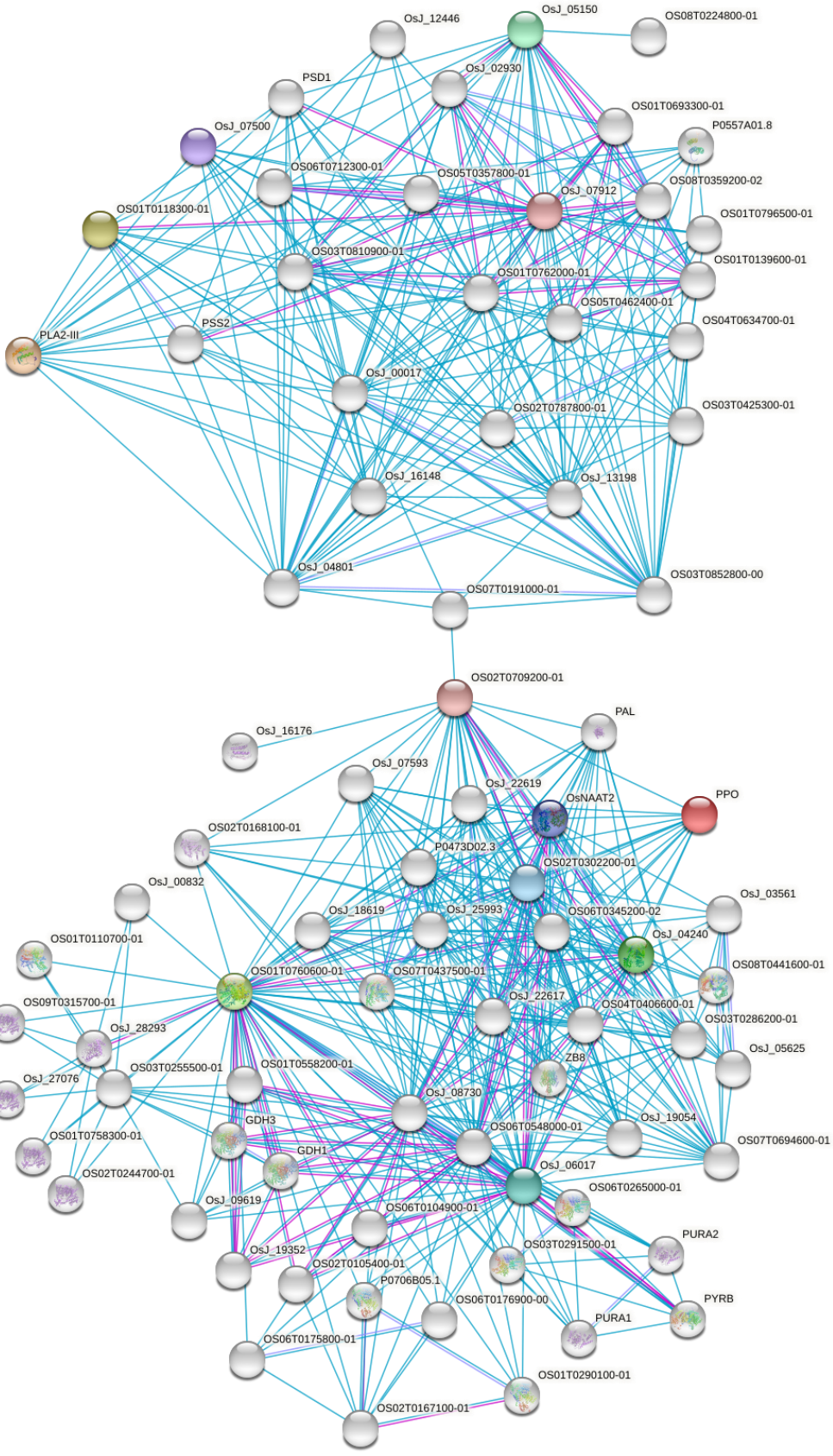


Figure 3. Protein-Protein Interaction prediction between OsPLA2-III and OsPPO proteins

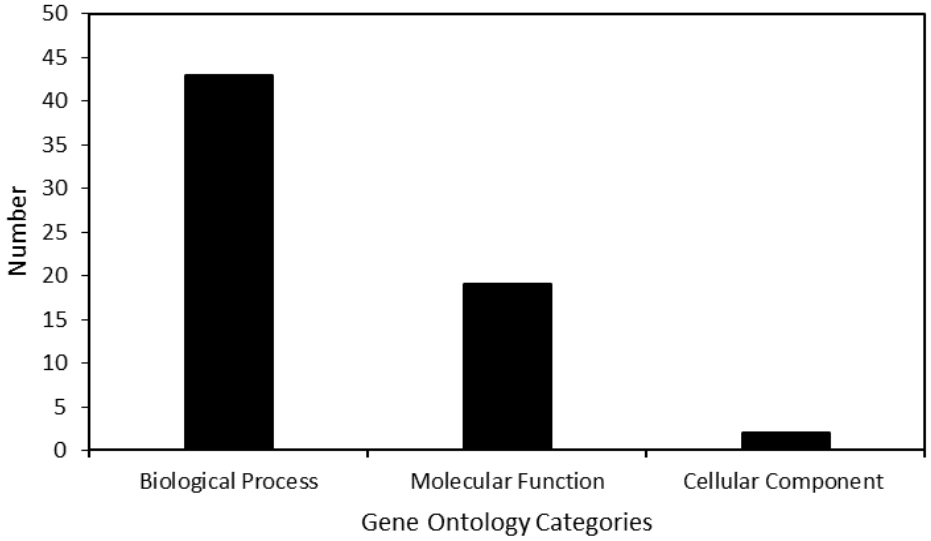


Figure 4. Gene ontology analysis based on the protein-protein interaction between OsPLA2-III and OsPPO proteins

two proteins. The depicted pattern in the network interaction of the proteins suggests that they both work in the tolerance mechanism against Fe toxicity stress through lipid signaling.

The role of OsPLA2-III in the tolerance mechanism of rice against Fe toxicity

OsPLA2-III gene is an encoded Phospolipase A2 (PLA2) and has a role in the lipid signaling process that activates the defense-related genes in plants [4, 21]. In rice, this gene (phospholipase A2 homolog 3-like) is located on chromosome 3.

Lipid products that are released by plants are suggested as bioactive compounds for cell signaling in relation to growth, development, and biotic and abiotic stimulus [22]. Furthermore, PLA2 is reported as one of the key enzymes of linoleic (map00591) and linolenic acid (map00592) biosynthesis. Phospholipase A2 enzyme is involved as a catalyst in the Ca-dependent hydrolysis mechanism of the 2-acyl group of 3-sn-phosphoglycerides. This process releases lysophospholipids (LPLs) and free fatty acid from phospholipid membranes as hormones and other external stimuli responses [21].

The pattern of the expression level of *OsPLA2-III* gene under Fe-toxicity treatment until 5 DAT (Figure 2a) suggests that a high concentration of free fatty acid was released from phospholipid membranes. Furthermore, this released lipid had a role in activating the Fe toxicity tolerance-related genes and the interaction network of *OsPLA2-III* associated proteins, as suggested in Figure 3.

A previous study showed that linoleic and linolenic acids are suggested to have a role as metabolite marker candidates for rice response to Fe toxicity stress through the maintenance process of the cell membranes during this stress [12]. Furthermore, the phospholipase activation indicates the beginning of the releasing process of important molecules for the defense system, such as oxylipine, jasmonic acid, and second messenger (phosphatidic acid (PA)) [23].

Phospholipase and phospholipid-derived compounds have an important role in modulating the plant defense system by activating various important processes, such as ROS production, protein-kinase pathway, Ca^{2+} signaling, hormones, and defense-related genes.

Role of OsPPO in the tolerance mechanism of rice against Fe toxicity

The expression level of *OsPPO* gene, which encodes polyphenol oxidase enzyme, contributed to the tolerance strategies of rice against Fe toxicity. The increase in the expression level in rice var. Pokkali, which concomitant with the decrease in the leaf bronzing level and increase in the chlorophyll content, indicated the tolerance to Fe toxicity (Table 1, Figure 1, and 2b). As reported previously, *OsPPO* gene plays a role in biotic and abiotic stress defence systems. Based on this study, the gene shows a complex interaction

network to defense stress-related genes under Fe toxicity conditions (Figure 3).

Polyphenol oxidases (PPOs) a metalloenzyme (copper metalloprotein) has been responsible for plant defence system and post-harvest reduction [7]. *OsPPO* gene is involved in the tolerance mechanism of *Arabidopsis thaliana* under drought and salinity stresses. This was indicated by the increase of GUS promoter of the *OsPPO* gene expression level with increasing stress levels [24].

The *OsPPO* gene encodes polyphenol oxidase, which is suggested as one of the antioxidant enzymes released due to Fe toxicity stress in this study. Furthermore, enzymatic ROS detoxification activities in leaves using polyphenol oxidase (PPO) is a potential biological marker for Fe tolerance level of *Vicia alba* under drought stress [25].

Haque et al. [26] showed high activity of *OsPPO* gene in the *Basella alba* until 48 hours after the low-temperature treatment. An increase in PPO enzyme also showed in radish (*Raphanus sativus*) after Pb stress of 25-500 ppm [27].

Conclusion

The expression level of *OsPLA2-III* and *OsPPO* genes were upregulated under Fe toxicity conditions. Thus, both genes are suggested to be involved in the tolerance mechanism against Fe toxicity stress though the lipid signaling mechanism. This finding would be useful as information to develop new molecular markers for the breeding program, especially to improve the Fe toxicity tolerance in rice.

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