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Differential antioxidant defense and detoxification mechanisms in photodynamically stressed rice plants treated with the deregulators of porphyrin biosynthesis, 5-aminolevulinic acid and oxyfluorfen

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ABSTRACT

This study focuses on differential molecular mechanisms of antioxidant and detoxification systems in rice plants under two different types of photodynamic stress imposed by porphyrin deregulators, 5-aminolevulinic acid (ALA) and oxyfluorfen (OF). The ALA-treated plants with white necrosis exhibited a greater decrease in photochemical quantum efficiency, F_v/F_m, as well as a greater increase in activity of superoxide dismutase, compared to the OF-treated plants. By contrast, the brown necrosis in OF-treated plants resulted in not only more widely dispersed H_2O_2 production and greater increases in H_2O_2-decomposing enzymes, catalase and peroxidase, but also lower ascorbate redox state. In addition, ALA- and OF-treated plants markedly up-regulated transcript levels of genes involved in detoxification processes including transport and movement, cellular homeostasis, and xenobiotic conjugation, with prominent up-regulation of serine/threonine kinase and chaperone only in ALA-treated plants. Our results demonstrate that different photodynamic stress imposed by ALA and OF developed differential actions of antioxidant enzymes and detoxification. Particularly, detoxification system may play potential roles in plant protection against photodynamic stress imposed by porphyrin deregulators, thereby contributing to alleviation of photodynamic damage.

1. Introduction

Plants use different forms of porphyrins as an electron carrier, signaling factor, or catalyst for redox reactions. The biosynthesis of porphyrin occurs through several steps where the formation of 5-aminolevulinic acid (ALA) is the first committed intermediate and protoporphyrin IX (Proto IX) is the last intermediate in the common pathway before separating into heme and chlorophyll branch [1,2]. Many intermediates in the porphyrin biosynthetic pathway, such as Proto IX and protochlorophyllide (Pchlide), interact with molecular oxygen in the presence of light to form reactive oxygen species (ROS), which is harmful to cells and causes the peroxidation of membrane lipids [3,4].

ALA applied at high concentrations acts as porphyrin deregulator because of abnormal accumulation of porphyrin intermediates which severely damaged treated plants when exposed to light [5,6]. Oxyfluorfen (OF) is an inhibitor of protoporphyrinogen oxidase (PPO) which catalyzes the step leading to the formation of Proto IX from protoporphyrinogen IX (Protogen IX) [7]. This inhibition results in the accumulation of Protogen IX which diffuses to the cytoplasm and is oxidized to Proto IX via peroxidase-like enzymes in membrane. Cytoplasmic Proto IX is a potent photosensitizer resulting in the formation of ROS, causing cell death [8]. Porphyrin biosynthesis and degradation are carefully adjusted to the cellular requirements, reflecting the different needs under varying stress conditions including drought and photodynamic stress [3,9,10]. There is a certain concentration threshold of photosensitizing porphyrin(ogen)s per light interval, below which plastids with their highly efficient antioxidative defense system can keep these metabolites in their reduced and therefore non-toxic state as well as sufficiently detoxify basal levels of photosensitization products [11]. Once this limit is exceeded, excited porphyrins tend to spread into other cellular compartments that are less well protected against their photodynamic action.

To detoxify oxidative stress, plants exhibit complementary protective responses including production of antioxidants system in the cell [12,13] as well as chaperone signaling and transcriptional activation [14,15]. The extent of stress-induced damage can be

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attenuated by the action of the cell's antioxidant systems, including ascorbate (Asc), glutathione (GSH), and enzymes capable of scavenging ROS [13,16]. Key examples of antioxidant enzymes are superoxide dismutase (SOD) and ascorbate peroxidase (APX) that disproportionate o_2^- and catalyze the conversion of H_2O_2 to water, respectively [13]. Peroxidase (POD) and catalase (CAT) are the main enzymes involved in H_2O_2 removal. In addition to the antioxidant network, plants must mount specific and coordinated defense mechanisms for survival under adverse conditions. One such mechanism is the capability for metabolizing xenobiotics, i.e. herbicides [17]. Plants treated with xenobiotics induce the expression of genes involved in plant defense and detoxification, such as glutathione *S*-transferases (GSTs) and cytochrome P450s (P450s) [18–20].

In our previous study, rice plants treated with ALA or OF exhibited different herbicidal symptoms as characterized by white and brown necrosis, respectively, resulted from differentially perturbed porphyrin biosynthesis [6]. In this study, we attempted to clarify differential responses not only in oxidative status, but also in molecular mechanisms of antioxidant system between the two different types of photodynamic stress, white and brown necrosis. We have also examined alterations in detoxification gene expression of the stressed plants to fulfill physiological purposes related to the inevitable photodynamic stress accompanying treatment with ALA and OF. Our results suggest the coordinated defense mechanisms of antioxidative and detoxification systems for plant protection against photodynamic stress imposed by porphyrin deregulators.

2. Material and methods

2.1. Plant growth and treatment of porphyrin deregulators

Germinated seeds of rice plants (*Oryza sativa* cv. Dongjin) were sown in pots which were filled with commercial greenhouse compost and were grown for 3 weeks in a greenhouse at 28 °C–30 °C. Three days before treatment of porphyrin deregulators, they were transferred to a growth chamber maintained at day/night temperatures of 28 °C/25 °C under a 14-h-light/10-h-dark cycle with a 200 μmol m^{-2} s^{-1} photosynthetic photon flux density. For the foliar application, three-week-old plants were sprayed with 5 mM 5-aminolevulinic acid (Fluka) or 50 μM commercial oxyfluorfen (Goal), which develops similar degree of dehydration in the treated plants. Control plants were treated with solvent only (30% acetone and 0.01% Tween 20). Rice plants were exposed to irradiation (14-h day/10-h night) for 2 days after 12-h dark incubation following either ALA or OF treatment. Parts of the youngest, fully developed leaves from the treated plants after 6 h and 30 h of illumination were taken for experiments.

2.2. In vivo detection of H_2O_2

H_2O_2 was visually detected in the leaves using 3,3-diaminobenzidine (DAB) [21]. The leaves were cut with a razor blade and incubated in a 1 mg ml^{-1} solution of 3,3-diaminobenzidine (DAB) (pH 3.8) for 4 h in light at 25 °C. The experiment was terminated by boiling the leaves in ethanol for 10 min. This treatment decolorized the leaves with the exception of the deep-brown polymerization product produced by the reaction of DAB with H_2O_2.

2.3. Measurement of photosynthetic activity

Chlorophyll *a* fluorescence was measured *in vivo* using a pulse amplitude modulation fluorometer (Handy PEA; Hansatech Instruments) after dark adaptation for 20 min. The minimal fluorescence yield, F_o, was obtained upon excitation with a weak measuring beam from a pulse light-emitting diode. The maximal fluorescence yield, F_m, was determined after exposure to a saturating pulse of white light to close all reaction centers. The ratio of F_v to F_m, representing the activity of photosystem II (PSII), was used to assess the functional damage to the plants.

2.4. Determination of ascorbate

For ascorbate (Asc) assay, tissues were homogenized in 5% metaphosphoric acid and centrifuged at 13,000 *g* for 15 min [22]. To measure total Asc the reduced Asc plus the oxidized dehydroascorbate (DHA) and the amount of reduced Asc, we mixed 100 μl of the supernatant with 250 μl of 0.15 M K_2HPO_4 buffer (pH 7.4) in the presence of either 50 μl of 10 mM dithiothreitol (DTT, for total Asc) or the same volume of water (for the reduced Asc). Samples were incubated for at least 10 min at room temperature before 50 μl of 0.5% *N*-ethylmaleimide was added. A color-developing solution [22] was added to each of the above mixtures. These were vigorously mixed and incubated at 37 °C for 60 min before absorbance at 525 nm was measured.

2.5. Assays for antioxidant enzymes

Leaves (0.25 g) were macerated to fine powder in a mortar under liquid N_2. Soluble proteins were extracted by homogenizing the powder in 2 ml of 100 mM potassium phosphate buffer, pH 7.5, containing 2 mM EDTA, 1% PVP-40, and 1 mM phenylmethylsulfonyl fluoride. Equal amounts of protein were electrophoresed on 10% nondenaturing polyacrylamide gels at 4 °C for 1.5 h at a constant current of 30 mA. For the ascorbate peroxidase (APX) activity, gels were soaked in 50 mM potassium phosphate buffer, pH 7.0, containing 2 mM ascorbate for 30 min and stained as described in Rao et al. [23]. The CAT activity was detected by incubating the gels in 3.27 mM H_2O_2 for 25 min and staining them in a solution of 1% potassium ferricyanide and 1% ferric chloride for 4 min [24]. The staining of peroxidase (POD) isozymes was achieved by incubating gels in sodium citrate buffer (pH 5.0) containing 9.25 mM *p*-phenylenediamine and 3.92 mM H_2O_2 for 15 min [25]. Gels were stained for SOD isoforms by soaking in 50 mM potassium phosphate (pH 7.8) containing 2.5 mM nitroblue tetrazolium (NBT) in darkness for 25 min, followed by soaking in 50 mM potassium phosphate (pH 7.8) containing 28 mM NBT and 28 μM riboflavin in darkness for 30 min. The gels were then exposed to light for 30 min [23].

2.6. RNA extraction and qRT-PCR

Total RNA was prepared from leaf tissues using TRIZOL Reagent (Invitrogen), and 5 μg of RNA from each sample was used for the reverse transcription reaction (SuperScript III First-Strand Synthesis System, Invitrogen). Subsequently, 50 ng of cDNA was used for qRT-PCR analysis. The qRT-PCR analysis was carried out with the 7300 Real-Time PCR system (Applied Biosystems) using Power SYBR Green PCR Master Mix (Applied Biosystems) and specific primers for genes (Supplemental Table S1). The qRT-PCR program consisted of 2 min at 50 °C, 10 min at 95 °C, and 40 cycles of 15 s at 95 °C and 1 min at 60 °C. A melting curve analysis was performed after every PCR reaction to confirm the accuracy of each amplified product. All reactions were set up in triplicate. The sample of control was used as the calibrator, with the expression level of the sample set to 1. Actin was used as the internal control.

3. Results and discussion

3.1. Altered oxidative metabolism in rice plants treated with ALA and oxyfluorfen

Foliar application of the porphyrin deregulators, 5 mM ALA and 50 μM OF, which cause plants to accumulate their own porphyrin intermediates resulted in white and brown necrotic symptoms, respectively, in rice plants after 2 days of the treatments (Fig. 1A). Brown necrosis in OF-treated plants resulted from the effect of peroxidation due to the great accumulation of Proto IX in cytoplasm and plasma membrane, whereas photodynamic degradation via abnormal accumulation of Proto IX and Pchlide in chloroplasts caused white necrosis due to bleaching effect in ALA-treated plants [6]. When expose to light, these excess porphyrins were photosensitized [3,7,8]. If excited porphyrins are left unquenched, they can form highly toxic radicals [26] and may endanger the plant cell.

To investigate whether these porphyrin deregulators influence ROS generation in the treated tissues, untreated leaves as well as ALA- and OF-treated leaves were incubated with DAB for the detection of H_2O_2 production, which is a marker of oxidative stress in plant tissues. Leaves from both ALA- and OF-treated plants slightly increased H_2O_2 production after 1 day of the treatments and then dramatically increased it after 2 days, whereas control leaves did not show any marked production of H_2O_2 (Fig. 1B). Noticeably, brown (in the web version) spots representing H_2O_2 localization were more widely dispersed throughout the leaves of OF-treated plants, as compared to those of ALA-treated plants. Effect of ALA and OF on photosynthetic performance was verified by measuring changes in photochemical quantum efficiency, F_v/F_m, a trait positively correlated with the organization and vitality of PSII. The photodynamic damage caused by foliar application of ALA and OF led to a reduction in F_v/F_m after 2 days of the treatments, as compared to control plants (Fig. 1C). The greater reduction of F_v/F_m in ALA-treated plants (Fig. 1C) may be resulted from bleaching due to the greater photodynamic degradation of porphyrin intermediates [6], compared to OF-treated plants. Under photodynamic stress imposed by ALA and OF, tight control of porphyrin biosynthesis prevented accumulation of toxic metabolic intermediates by down-regulation of their biosynthesis, but largely by photodynamic degradation [6], thereby alleviating photodynamic damage. However, this regulatory mechanism of porphyrin metabolism is not sufficient to overcome the photodynamic stress, and thus the plants necessitate other components of protective process.

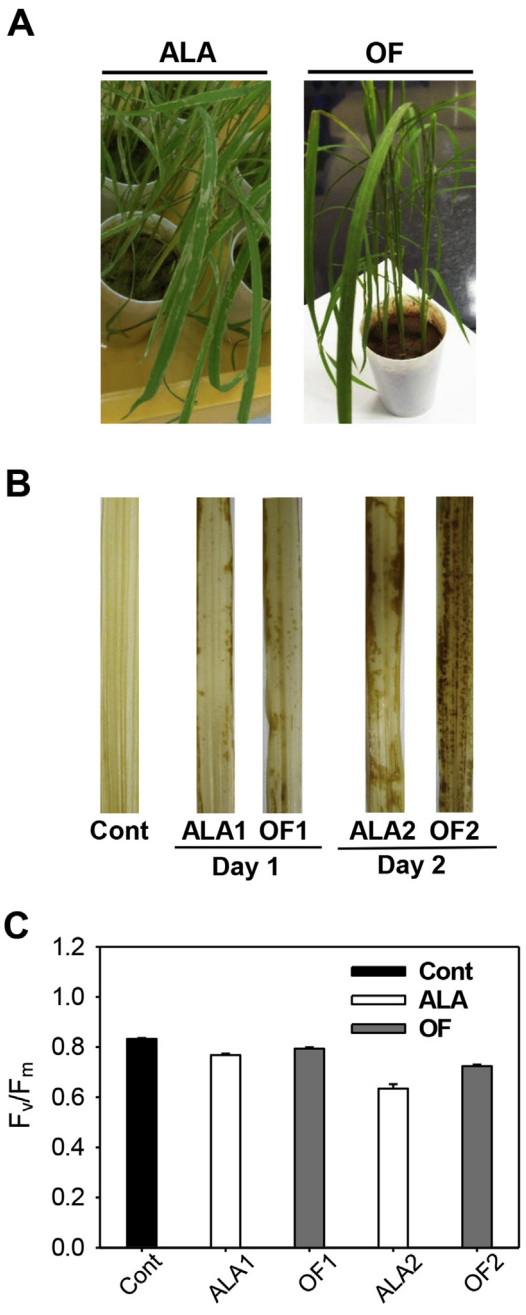

Fig. 1. Necrotic symptoms and oxidative metabolism of rice plants in response to ALA and OF treatment. (A) Necrotic symptoms on leaves. (B) H_2O_2-DAB-staining in leaves. The production of H_2O_2 was visually detected by browning of leaf veins after DAB incubation. (C) Photosynthetic performance. The efficiency of PSII photochemistry, F_v/F_m, was used to assess the functional damage to the plants. Three-week-old rice plants were sprayed with 5 mM ALA or 50 μM OF, placed in darkness for 12 h to allow absorbance, and then illuminated for either 6 h or 30 h. Cont, control; ALA1 and OF1, 1 day after ALA and OF treatment, respectively; ALA2 and OF2, 2 days after ALA and OF treatment, respectively. The data represent the mean ± SE of six replicates from two independent experiments.

3.2. Effect of ALA and oxyfluorfen on cellular redox state and activities of antioxidant enzymes

The steady-state levels of H_2O_2, $^1O_2$, o_2^-, and the hydroxyl radical depend on the balance between generation and removal, which is facilitated by the ROS-scavenging system including both non-enzymatic and enzymatic constituents in plant cells [12,13]. To test whether porphyrin deregulators, ALA and OF, modify cellular redox state, Asc content was used as a biochemical marker of cell redox state. After 2 days of ALA and OF treatment, the content of Asc and the redox state of Asc (estimated as Asc × 100/DHA + Asc) significantly decreased in the treated plants, while the content of DHA increased (Fig. 2). The OF-treated plants exhibited a greater increase in DHA content, thereby leading to a greater decline in the redox state of Asc. During stress, the pools of cellular Asc and GSH shift toward their oxidized forms, and the redox state of Asc and GSH is decreased [13,16].

We also monitored alterations in isozyme profiles of antioxidant enzymes in response to foliar application of ALA and OF. The activities of all antioxidant enzymes examined were greatly increased in ALA- and OF-treated plants (Fig. 3A). Noticeable increases in staining activities of Cu/Zn-SOD isozymes 1 and 3 were observed only in ALA-treated plants after 2 days of the treatment. Both ALA- and OF-treated plants increased activities of Mn-SOD isozyme 2 after 2 days of the treatments, with a greater increase in ALA-treated plants (Fig. 3A). During oxidative stress, cells express a set of H_2O_2-decomposing enzymes, namely CAT, APX, and POD [12,13].

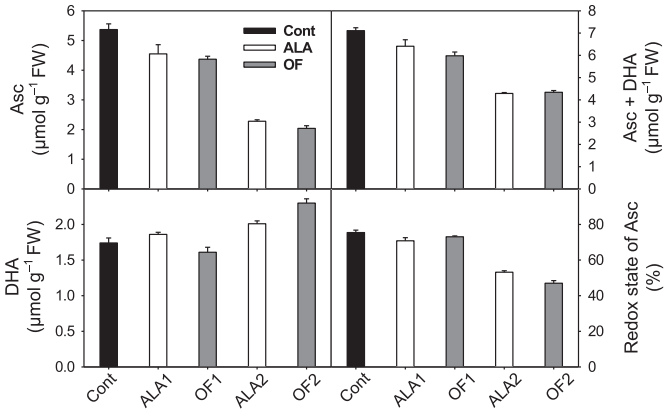

Fig. 2. Cellular redox state in leaves of rice plants exposed to ALA and OF treatment. Asc, ascorbic acid; DHA, dehydroascorbate; redox state of ascorbate: estimated as Asc × 100/Asc_t, where Asc_t = DHA + Asc. The plants were subjected to the same treatments as in Fig. 1. Treatment notations are the same as in Fig. 1. The data represent the mean ± SE of six replicates from two independent experiments.

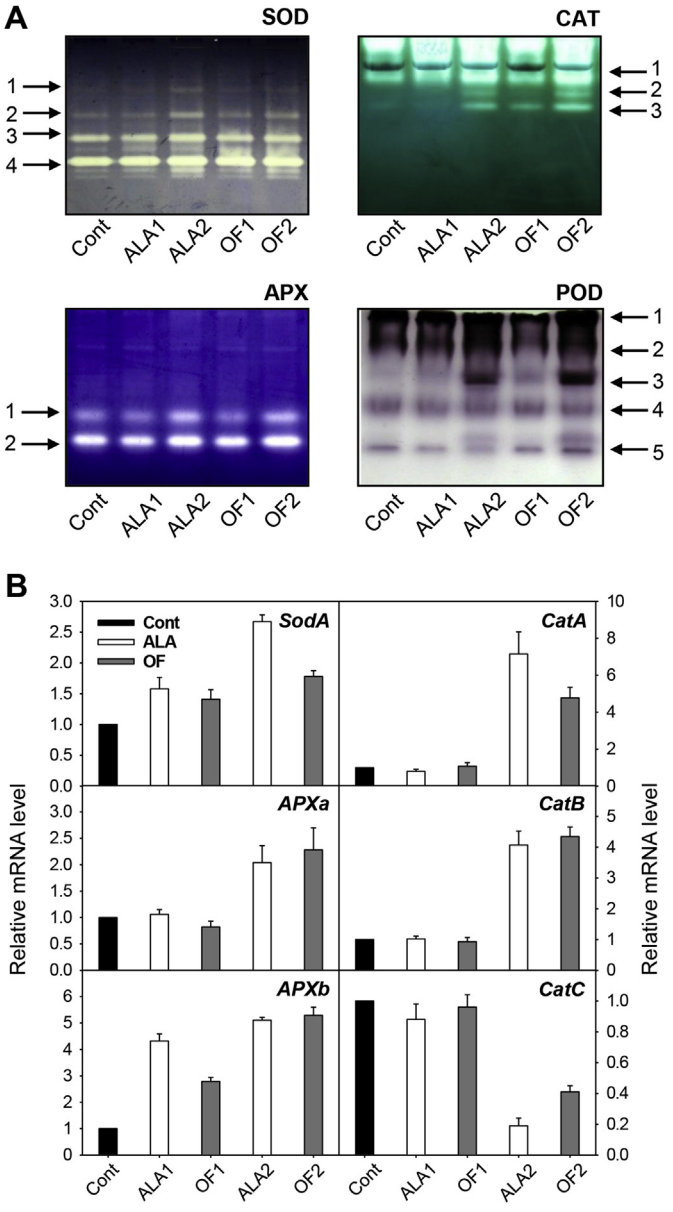

Fig. 3. The ALA- and OF-induced changes in the activities of ROS-scavenging enzymes. (A) Profiles of antioxidant isozymes. Non-denaturing activity gels were prepared and run as described in the method. Incubation of the gels with KCN or H_2O_2 prior to staining for SOD activity suggests that SOD-2 is a Mn-SOD, whereas SOD-1, SOD-3, and SOD-4 are Cu/Zn-SOD enzymes (data not shown). (B) Expression of genes encoding the ROS-scavenging enzymes. Total RNAs were purified from plants and reverse transcribed. The resultant cDNAs were used as templates for qRT-PCR using *Actin* as an internal control. The control was used for normalization, with the expression level of the sample set to 1. Error bars represent SE, and representative data from three independent experiments are presented. The plants were subjected to the same treatments as in Fig. 1. Treatment notations are the same as in Fig. 1.

The CAT isozyme 3 was detected only in OF-treated plants after 1 day of ALA and OF treatment, whereas OF-treated plants exhibited greater increases in staining activities of all three CAT isozymes than ALA-treated plants after 2 days of both treatments (Fig. 3A). In comparison to untreated controls, staining activities of APX and POD began to increase 2 days after ALA and OF treatment, especially with a greater increase of POD isozyme 3 in OF-treated plants than in ALA-treated plants.

In parallel with the increased activities of antioxidant enzymes, ALA- and OF-treated plants responded to oxidative stress by greatly up-regulating transcript levels of ROS-scavenging genes. The greater increase of *SodA* transcript in ALA-treated plants corresponded well to the stronger activity of SOD after 2 days of the treatment, compared to OF-treated plants (Fig. 3B). Transcript levels of *APXb* gradually increased during ALA and OF treatment, whereas transcript levels of *APXa*, *CatA*, and *CatB* began to increase 2 days after the treatments. In contrast to other ROS-scavenging genes, transcript levels of *CatC* decreased greatly 2 days after ALA and OF treatment. The prominent up-regulation of *CatA*, *CatB*, *APXa*, and *APXb* as well as the increased activities of hemoproteins, such as APX, CAT, and POD are in agreement with the up-regulation of *FC2* (*Fe-Chelatase2*) which is involved in heme synthesis under the photodynamic stress [6]. The balance between ROS production and antioxidant enzyme activities determines whether oxidative signaling or damage will occur [27]. In our study, activation of antioxidative pathways for detoxifying oxidative stress lacks capacity to eliminate adverse impacts of ROS on plants under the photodynamic damage caused by ALA and OF.

3.3. Porphyrin deregulators have a dramatic effect on expression of genes involved in plant defense and detoxification

To gain insight into the molecular mechanisms underlying the ALA- and OF-induced changes in detoxification responses, we used qRT-PCR to analyze the expression of genes with known regulatory roles in plant defense and detoxification. First, we examined transcript levels of genes encoding proteins that function in transport and movement. After 2 days of ALA and OF treatment, the treated rice plants exhibited drastic increases in transcript levels of genes encoding aquaporins, which are known as water channels and selectively conduct water molecules in and out of the cell [28], and pleitropic drug resistance (PDR) protein (Fig. 4). PDR is a subfamily of the ATP-binding cassette transporters and plays a role in plant defense against biotic and abiotic stresses [29]. In contrast, transcript levels of gene encoding Na^+/H^+ exchangers that are transporters playing a major role in cellular pH and Na^+ homeostasis [30], decreased in response to ALA and OF treatments.

The network of serine/threonine kinases in plant cells accepts information from receptors that sense environmental conditions and other external factors, and converts it into changes in metabolism and gene expression [31]. Transcript levels of gene encoding serine/threonine kinase receptor significantly up-regulated only in ALA-treated plants after 2 days of the treatment, but not in OF-

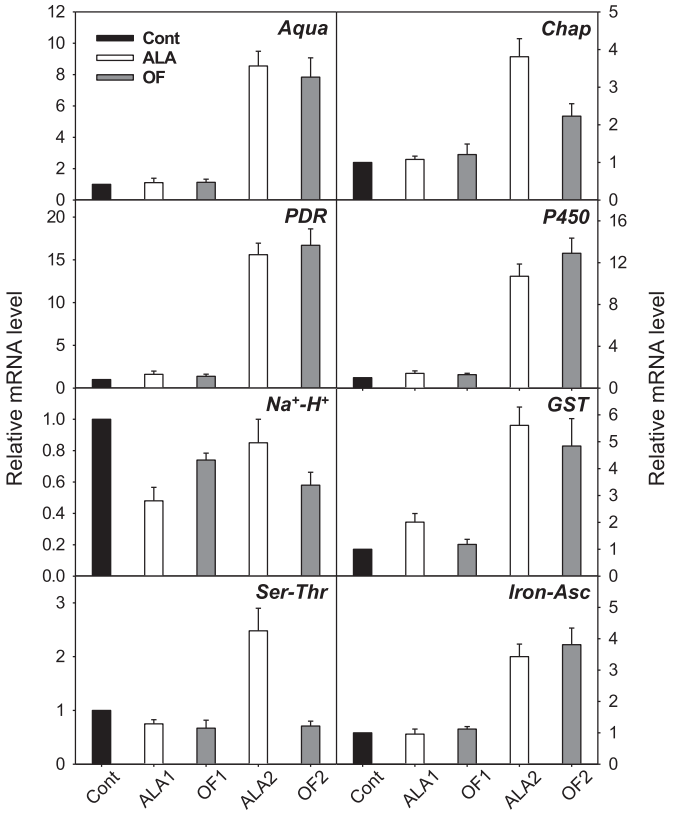

Fig. 4. The ALA- and OF-induced changes in expression of genes encoding the detoxifying proteins in leaves of rice plants. The plants were subjected to the same treatments as in Fig. 1. Treatment notations are the same as in Fig. 1. Aqua, aquaporin; Na^+–H^+, Na^+/H^+ exchangers; Ser–Thr, serine/threonine kinase receptor; Chap, chaperone; Iron-Asc, iron- and ascorbate family oxidoreductase. Total RNAs were purified from plants and reverse transcribed. The resultant cDNAs were used as templates for qRT-PCR using *Actin* as an internal control. The control was used for normalization, with the expression level of the sample set to 1. Error bars represent SE, and representative data from three independent experiments are presented.

treated plants (Fig. 4). This implicates a possible role of ALA as a signal mediator during photodynamic stress-mediated signaling pathway. The porphyrin intermediate Mg-Proto IX accumulates under oxidative stress conditions, is exported from the chloroplast, and transmits the plastid signal, which is vital to plants during stress responses [26,32]. Transcript levels of gene encoding chaperones, which can protect plants against stress by reestablishing normal protein conformation and cellular homeostasis [33], exhibited a greater increase in ALA-treated plants, compared to OF-treated plants (Fig. 4).

Groups of proteins which function in xenobiotic metabolism also contributed to detoxify the photodynamic stress caused by ALA and OF. The cytochrome P450s are hemoproteins that have important roles in the synthesis of lignin, pigments, defense compounds, and signaling molecules in plants [20]. Transcript levels of two genes encoding P450 and GST slightly increased 1 day after ALA and OF treatment and markedly increased 2 days after the treatments (Fig. 4). For the purpose of detoxification, plant GSTs attach GSH to electrophilic xenobiotics, which tag them for vacuolar sequestration [19]. After 2 days of ALA and OF treatment, plants greatly up-regulated transcript levels of gene encoding iron- and ascorbate family oxidoreductase (Fig. 4), which catalyzes the transfer of electrons from one molecule to another. Although the precise role of these detoxifying genes in the photodynamic stress caused by ALA and OF has yet to be established, all these mechanisms, which are regulated at the molecular level, enable plants to thrive under stress.

Under the two different types of photodynamic stress imposed by ALA and OF treatment, rice plants substantially increased differential antioxidant defense and detoxification mechanisms. The ALA-treated plants resulted in the greater reduction in F_v/F_m as well as greater increases in expression of SOD than the OF-treated plants, indicating that higher photooxidative stress is imposed on white necrosis of ALA-treated plants. On the other hand, the brown necrosis in OF-treated plants exhibited not only more widely dispersed H_2O_2 production and accompanying increases in H_2O_2-decomposing enzymes, CAT and POD, but also lower Asc redox status compared to those of ALA-treated plants. Both ALA- and OF-treated plants markedly up-regulated transcript levels of genes involved in various detoxification processes including transport and movement, cellular homeostasis, and xenobiotic conjugation, with greater transcript levels of serine/threonine kinases and chaperone in ALA-treated plants. Our results provide evidence that the coordinated defense mechanisms of both antioxidative and detoxification systems, particularly in relation to hemoproteins, work together to alleviate photodynamic stress imposed by porphyrin deregulators.

Conflict of interest

None.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.02.125.

Transparency document

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