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Perturbed porphyrin biosynthesis contributes to differential herbicidal symptoms in photodynamically stressed rice (*Oryza sativa*) treated with 5-aminolevulinic acid and oxyfluorfen

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ABSTRACT

This paper focuses on the molecular mechanism of deregulated porphyrin biosynthesis in rice plants under photodynamic stress imposed by an exogenous supply of 5-aminolevulinic acid (ALA) and oxyfluorfen (OF). Plants treated with 5 mM ALA or 50 μM OF exhibited differential herbicidal symptoms as characterized by white and brown necrosis, respectively, with substantial increases in cellular leakage and malondialdehyde production. Protoporphyrin IX accumulated to higher levels after 1 day of ALA and OF treatment, whereas it decreased to the control level after 2 days of ALA treatment. Plants responded to OF by greatly decreasing the levels of Mg–protoporphyrin IX (MgProto IX), MgProto IX methyl ester, and protochlorophyllide to levels lower than control, whereas their levels drastically increased 1 day after ALA treatment and then disappeared 2 days after the treatment. Enzyme activity and transcript levels of *HEMA1, GSA* and *ALAD* for ALA synthesis greatly decreased in ALA- and OF-treated plants. Transcript levels of *PPO1, CHLH, CHLI*, and *PORB* genes involving Mg–porphyrin synthesis continuously decreased in ALA- and OF-treated plants, with greater decreases in ALA-treated plants. By contrast, up-regulation of *FC2* and *HO2* genes in Fe–porphyrin branch was noticeable in ALA and OF-treated plants 1 day and 2 days after the treatments, respectively. Decreased transcript levels of nuclear-encoded genes *Lhcb1, Lhcb6*, and *RbcS* were accompanied by disappearance of MgProto IX in ALA- and OF-treated plants after 2 days of the treatments. Under photodynamic stress imposed by ALA and OF, tight control of porphyrin biosynthesis prevents accumulation of toxic metabolic intermediates not only by down-regulation of their biosynthesis but also by photodynamic degradation. The up-regulation of *FC2* and *HO2* also appears to compensate for the photodynamic stress-induced damage.

1. Introduction

The porphyrin biosynthetic pathway provides the vital cofactors and pigments for chlorophylls, heme, siroheme, and phytochromobilin. The biosynthesis of porphyrin in all living cells occurs through several steps where the formation of 5-aminolevulinic acid (ALA) is the first committed intermediate [1]. ALA is formed in a three-step reaction including the ligation of glutamate to $tRNA^{Glu}$ catalyzed by glutamyl-tRNA synthetase, the reduction of glutamate to glutamate-1-semialdehyde by glutamyl-tRNA reductase (GluTR) and a final transamination step mediated by glutamate-1-semialdehyde aminotransferase [2,3]. Protoporphyrinogen oxidase (PPO), which catalyzes the oxidation of protoporphyrinogen IX (Protogen IX) to protoporphyrin IX (Proto IX), is the last enzyme before the branch in the porphyrin biosynthetic pathway, and its product, Proto IX, is directed to the magnesium (Mg) and iron (Fe) branches for chlorophyll and heme biosynthesis, respectively [4].

Several photobleaching herbicides, including diphenyl ethers, oxaldiazoles, and *N*-phenyl imides, have been found to cause the accumulation of photodynamic porphyrins [5]. ALA is the substrate of porphyrin and a peroxidizing herbicide oxyfluorfen (OF) inhibits PPO activity. High concentrations of ALA acted as porphyrin deregulator because of high accumulation of porphyrin intermediates which severely damaged treated plants when exposed to light [6,7]. However, ALA and ALA-based chemicals [8] are not used as field herbicides. At low concentration ALA acts as a promotive effect on the growth and photosynthesis of crops and vegetables [9,10] as well as a protective function against oxidative stress [11,12]. The inhibition of PPO activity by OF [13] results in the accumulation of Protogen IX which diffuses to the cytoplasm and is oxidized to Proto IX via peroxidase-like enzymes in the membrane [14,15]. However, there is little known about the differential mechanism of molecular deregulation in porphyrin biosynthesis induced by ALA and OF.

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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 [16–19]. Porphyrin biosynthesis and degradation are carefully adjusted to the cellular requirements, reflecting the different needs under varying stress conditions [20–23]. Water stress controls metabolites of the porphyrin biosynthetic pathway through their scavenging to cope with excited-state dynamics of porphyrins in the cell, consequently attenuating the photodynamic stress imposed by drought [24]. Porphyrin intermediate biosynthesis may provide signals to control expression of nuclear genes in response to metabolic activity in chloroplasts [25–27]. Stress-induced perturbations of the porphyrin biosynthetic pathway trigger large changes in nuclear gene expression, and specific accumulation of MgProto IX and MgProto IX methyl ester (ME) was demonstrated in numerous studies to coincide with these changes in nuclear gene expression [28–30]. By contrast, other reports have demonstrated that changes in MgProto IX accumulation do not correlate with changes in nuclear gene expression [31,32].

In this study, we examined the differential herbicidal symptoms and molecular mechanism of deregulated porphyrin biosynthesis in rice plants under photodynamic stress imposed by either ALA or OF treatment. Plants treated with ALA that influences upstream of porphyrin biosynthetic pathway showed different changes in porphyrin metabolism as well as photodynamic damage, compared to plants treated with OF that influences downstream of the pathway. Our results suggest that differential control of porphyrin biosynthesis is tightly related to characteristic symptoms of the treated plants by ALA and OF. The association of nuclear photosynthetic gene expression with porphyrin intermediates under photodynamic stress was also discussed.

2. Materials and methods

2.1. Plant growth and herbicidal treatment

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 ALA and OF treatment, 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 (PPFD). 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 a 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. Technical-grade OF (Gyungnong, Gyeongju, Korea) was used for cellular leakage measurement.

2.2. Cellular leakage

The rice leaf tissues were treated with ALA and OF as described previously by Lee et al. [33]. by cutting 4-mm leaf squares (0.1 g FW) with a razor blade and then placing them in a 6-cm diameter polystyrene Petri dish containing 5 mL of 1% sucrose and 1 mM MES (pH 6.5) with or without the herbicides, ALA and OF. The tissues were incubated with various concentrations of ALA and OF in a growth chamber at 25 °C in darkness for 12 h, and then exposed to continuous white light at 250 μmol m^{-2} s^{-1} PPFD for 24 h. Cellular leakage was determined periodically by the detection of electrolyte leakage into the bathing medium using a conductivity meter (Cole-Parmer Instruments) as described by Lee et al. [33].

2.3. Lipid peroxidation

Lipid peroxidation was estimated by the level of malondialdehyde (MDA) production using a slight modification of the thiobarbituric acid (TBA) method described by Buege and Aust [34]. The treated leaf tissues (0.1 g) were homogenized with a mortar and pestle in 5 mL of a solution of 0.5% (v/v) TBA in 20% trichloroacetic acid (TCA). The homogenates were centrifuged at 20,000 *g* for 15 min, and the supernatants were collected. The supernatants were heated in a boiling water bath for 25 min then cooled in an ice bath. Following centrifugation at 20,000 *g* for 15 min, the resulting supernatants were used for spectrophotometric determination of MDA. The absorbance at 532 nm for each sample was recorded and corrected for non-specific turbidity at 600 nm. The MDA concentration was calculated using a molar extinction coefficient of 156 mM^{-1} cm^{-1} following the formula: MDA (μmole g^{-1} FW) = $[(A_{532} - A_{600})/156] \times 10^3 \times$ dilution factor.

2.4. ALA-synthesizing capacity

ALA-synthesizing capacity was measured as described by Papenbrock et al. [35]. Leaf disks were incubated in 20 mM phosphate buffer (pH 6.9) containing 40 mM levulinic acid in the light for 6 h. Samples were homogenized, resuspended in 1 mL of 20 mM phosphate buffer (pH 6.9), and centrifuged at 10,000 *g*. The 500-μL supernatant was mixed with 100 μL ethylacetoacetate, boiled for 10 min, and cooled for 5 min. An equal volume of modified Ehrlichs reagent was added and the absorption of the chromophore was determined at 553 nm.

2.5. Porphyrin extraction and analysis

Porphyrins were extracted and analyzed following the method of Lermontova and Grimm [36]. Leaf tissue was ground in methanol:acetone:0.1 N NaOH (9:10:1, [v/v]) and the homogenate was centrifuged at 10,000 *g* for 10 min. Porphyrin was separated by HPLC using a Novapak C_{18} column (4-μm particle size, 4.6 × 250 mm, Waters) at a flow rate of 1 mL min^{-1}. Porphyrins were eluted with a solvent system of 0.1 M ammonium phosphate (pH 5.8) and methanol. The column eluate was monitored using a fluorescence detector (2474, Waters) at excitation and emission wavelengths of 400 and 630 nm for Proto IX, 440 and 630 nm for Pchlide, and 415 and 595 nm for MgProto IX and MgProto IX ME, respectively. All porphyrins were identified and quantified using authentic standards. The chlorophyll content was spectrophotometrically determined according to the method of Lichtenthaler [37].

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 (Supplementary 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 1 was used as the calibrator, with the expression level of the sample set to 1. Actin was used as an internal control.