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Resistance Mechanisms in Protoporphyrinogen Oxidase (PROTOX) Inhibitor-resistant Transgenic Rice

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We investigated the mechanism for conferring herbicide resistance in transgenic rice. Plants from Line M4 were resistant to PROTOX inhibitors and had yields similar to those from wild-type (WT) rice. *Myxococcus xanthus* PROTOX mRNA was abundantly expressed in the transgenic leaf tissues, and the *M. xanthus* PROTOX gene was stably transmitted into the T_4 generation. We detected a protein with a predicted molecular mass of 50 kD, equal to the weight of *M. xanthus* PROTOX, in M4 but not WT plants. Furthermore, several PROTOX-inhibitor herbicides -- acifluorfen, oxyfluorfen, carfentrazone-ethyl, and oxadiazon -- caused significant cellular leakage and lipid peroxidation in the WT, but not in the transgenics. Total PROTOX activity in untreated transformed rice was 17-fold higher than in the WT, with activity being inhibited in the latter genotype by 55%, 59%, 53%, or 60% as a result of treatment with acifluorfen, oxyfluorfen, carfentrazone-ethyl, or oxadiazon, respectively. However, PROTOX activities in transgenic rice were similar to their corresponding, untreated controls. The accumulation of Proto IX was 15- to 21-fold higher in the WT than in M4 when plants were treated with PROTOX inhibitors. In the former, their epicuticular wax and chloroplasts were severely damaged after oxyfluorfen treatment. The lack of damage in transformed plants suggests that M4 does not accumulate Proto IX, probably due to the production of herbicide-resistant chloroplastic and mitochondrial PROTOX.

Keywords: herbicide resistance mechanism, protoporphyrinogen oxidase (PROTOX), transgenic rice

A variety of PROTOX-inhibiting herbicides, including acifluorfen, oxyfluorfen, carfentrazone-ethyl, and oxadiazon, cause rapid peroxidative photobleaching and necrosis of green plant tissues (Duke et al., 1991; Scalla and Matringe, 1994). Their target site of action is protoporphyrinogen oxidase (PROTOX, EC 1.3.3.4), which catalyzes the oxidation of protoporphyrinogen IX (Protogen IX) to protoporphyrin IX (Proto IX) (Duke and Rebeiz, 1994; Matringe et al., 1989; Witkowski and Halling, 1989). This photoactive Proto IX can generate membrane-damaging radicals upon exposure to light and oxygen (Kouji et al., 1988; Lehnen et al., 1990). Inhibition of PROTOX activity results in a buildup of Protogen IX, which leaks from the chloroplast to the cytoplasm, wherein it rapidly oxidizes to Proto IX (Becerril and Duke, 1989; Jacobs et al., 1991; Lee et al., 1993). Proto IX cannot re-enter the chloroplast because of its high lipophilic nature, eventually leading to its partitioning into the membranes (Lehnen et al., 1990). Because Proto IX cannot interact with Mg- and Fe-chelatase located in the chloroplasts or mitochondria, it does not get utilized in the chlorophyll or heme synthesis pathway (Matringe et al., 1989). Proto IX in the cytoplasm generates highly reactive singlet oxygen in the presence of light and molecular oxygen, causing rapid peroxidation of the cell membrane and, ultimately, lethal cell damage (Becerril and Duke, 1989; Jacobs et al., 1991).

A large number of PROTOX genes have been cloned from prokaryotes and eukaryotes. Almost all eukaryotic PROTOX genes, with the exception of a spinach PROTOX II (dually targeted to the chloroplasts and mitochondria), feature single-targeting proteins that are destined to be expressed at one sub-cellular site within either the chloroplasts or the mitochondria (Watanabe et al., 2001). In tobacco (*Nicotiana tabacum*), two forms of PROTOX that share only 27% amino acid identity are independently targeted to different cellular compartments -- chloroplasts and mitochondria (Lermontova et al., 1997). In contrast, mammals possess only a mitochondrial form of PROTOX that is imported into the inner membrane (Ferreira et al., 1988; Morgan et al., 2004). To produce transgenic plants with resistance to PROTOX-inhibiting herbicides, chloroplastic expression of PROTOX genes from *Bacillus subtilis* and *Arabidopsis thaliana* has been used to transform tobacco (Choi et al., 1998; Lermontova and Grimm, 2000) and rice (Ha et al., 2004; Lee et al., 2000). These transgenic plants are resistant to PROTOX-inhibiting herbicides, such as oxyfluorfen and acifluorfen. Resistance to PROTOX inhibitors can also be conferred via mitochondrial PROTOX, as shown by soybean cell lines with a 9-fold higher level of mitochondrial PROTOX activity compared with non-resistant cells (Warabi et al., 2001). However, the above approaches lead to only low levels of herbicide resistance. This can be attributed to the localization of PROTOX in two different sub-cellular compartments. Because both PROTOX isozymes are sensitive to PROTOX-inhibiting herbicides (Jacobs et al., 1991; Matringe et al., 1989), the overexpression of either by itself is not a suitable tool for generating transgenic plants that will be protected against herbicide attack. However, when a *Myxococcus xanthus* PROTOX gene, without an additional plastidal transit sequence, is simultaneously expressed in both the chloroplasts and mitochondria of rice, the resulting transgenic plants show greatly increased herbicide resistance (Jung et al., 2004).

Therefore, the three objectives of our research were to a) confirm whether this dual expression of the *Myxococcus xanthus* PROTOX gene could confer a high degree of resistance to PROTOX inhibitors (acifluorfen, oxyfluorfen, carfentrazone-ethyl, and oxadiazon), b) monitor changes in the epidermal and ultrastructural characteristics of transgenic

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and wild-type rice plants in response to oxyfluorfen, and c) investigate the mechanism of resistance to PROTOX-inhibiting herbicides in transgenic rice.

MATERIALS AND METHODS

Plant Materials and Chemical Herbicides

Transgenic rice lines were created with the binary vector pGA1611:MP, which contains the hygromycin phosphotransferase gene as a selectable marker and the *M. xanthus* PROTOX (MP) gene. The resultant pGA1611 was transferred into *Agrobacterium tumefaciens* LBA4404, and *A. tumefaciens*-mediated transformation of rice was carried out as previously described (Jung et al., 2004) to produce Lines M1 through M8. Our herbicide-susceptible transgenic control (TC) was transformed only with binary vector pGA1611. These were all compared against a wild-type (WT) Korean rice cultivar, 'Dongjin'. The following chemical herbicides were tested here, using their commercial formulations: acifluorfen (Blazer; 590 g ai ha^{-1}), oxyfluorfen (Goal; 50 g ai ha^{-1}), carfentrazone-ethyl (Aim; 850 g ai ha^{-1}), and oxadiazon (Ronstar; 1270 g ai ha^{-1}).

General Growing Conditions

Seeds of WT and transgenic rice were soaked in water for 4 d at 25°C. After germination, they were sown in trays (60 cm × 30 cm × 3 cm) filled with commercial potting soil (Pungnong NPKO, Seoul, Korea), and placed in a greenhouse maintained at 30°C/25°C (day/night), 65%/80% relative humidity, and a photosynthetic photon flux (PPF) of 500 μmol m^{-2} s^{-1} under a 14-h photoperiod.

Confirmation of Hygromycin Resistance and Yield of Transgenic Lines at the T_3 Generation

To confirm hygromycin resistance and yield, we used a field that had long been a rice paddy before our experiments began, but which was kept fallow for five months after the last harvest. At 25 d after germination, seedlings from transgenic Lines M1 through M8 plus those of the TC and WT were transplanted into the field by hand, with 15 cm between plants and 30 cm between rows. Plots were 4 × 5 m, and we followed a randomized complete block design, with three replications. Nitrogen (N) fertilizer was applied at 110 kg N ha^{-1}, 50% of which was used before transplanting, 30% at the five-leaf stage, and 20% at panicle initiation. Before the transplanting began, phosphorus and potassium were added at 70 and 80 kg ha^{-1}, respectively. Other management practices were in accordance with standard methods for rice cultivation from the Rural Development Administration of Korea (RDA, 1998).

Six weeks after transplanting, two rice leaves per hill were harvested to test for hygromycin resistance. Leaf disks (100 mg) from the WT and transgenic plants were cultured in water containing hygromycin (50 μg mL^{-1}). These were placed in a growth chamber with conditions of 70% relative humidity, 25°C/25°C (day/night), and a 14-h photoperiod under fluorescent white light (250 μmol m^{-2} s^{-1} PPF). At 7 d after treatment (DAT), injury to the leaf tissue caused by hygromycin was evaluated visually. Samples from transgenic plants were resistant, remaining mostly green, while those from the WT were bleached or necrotized.

To determine grain yield, 22 hills per m^2 were harvested from three 1-m^2 quadrats per plot. All harvested plants were resistant to hygromycin. Yield variables, including panicles per plant, spikelets per panicle, mature grain (%), and 1000-grain weight (g), were recorded. Data were analyzed using the SAS (2000) ANOVA procedure, and means were separated by a Fisher's protected LSD test ($P = 0.05$).

Northern Blot Analysis

Total RNA (10 μg) was isolated from leaves of T_4 transgenic and WT plants with TRI reagent (Sigma-Aldrich Chemical Co., St. Louis, MO, USA), then fractionated on a 1% agarose gel containing formaldehyde, with 20 mM 3-(*N*-morpholino) propanesulfuric acid as the electrophoresis buffer. The gel was blotted onto a nylon membrane and hybridized with the *Myxococcus xanthus* PROTOX gene, radio-labelled with a Prime-It Kit (Stratagene) at 60°C in 0.25 M sodium phosphate buffer (pH 7.5), 7% SDS, 1% BSA, and 1 mM EDTA. After hybridization, the RNA blot was washed at 55°C, twice with 2 × SSC (1 × SSC; 0.15 M NaCl and 0.015 M sodium citrate; pH 7)/0.1% SDS, and twice with 0.2 × SSC/0.1% SDS.

Antibody Production and Immunoblotting

A polyclonal mouse antiserum was raised against the synthetic peptide IRNAAQLADALVAGNTSHA, which corresponds to the C-terminal 19 amino acids of *M. xanthus* PROTOX (Jung et al., 2004). Rice leaves were pulverized in a tissue grinder, and 100 mg was solubilized (Lermontova and Grimm, 2000) in 1 mL solubilization buffer (56 mM Na_2CO_3, 56 mM DTT, 2% SDS, 12% sucrose, and 2 mM EDTA). After the total protein was quantified, it was precipitated in 1.5 volumes of 100:1 methanol:acetic acid and 0.1 volume of 1 M NaCl before the pellet was dissolved in a loading buffer. Proteins were separated using 11% SDS-PAGE, then electro-blotted to polyvinylidene difluoride membranes. Immunodetection was performed according to standard procedures (Boehringer-Mannheim, Germany).

Electrolyte Leakage

Using 4-week-old plants, electrolyte leakage was measured from leaf tissues (100 mg) that were incubated in Petri dishes (6-cm diam.) containing 5 mL of 1 mM 2-(*N*-morpholino) ethanesulfuric acid (pH 6.5) with or without PROTOX-inhibiting herbicides -- acifluorfen, oxyfluorfen, carfentrazone-ethyl, and oxadiazon. Oxyfluorfen (97%), carfentrazone-ethyl (92.6%), and oxadiazon (95.6%) were dissolved in acetone while acifluorfen (98%) was dissolved in water. Their concentrations were adjusted to 0.01, 0.1, 1, 10, 100, or 1000 μM. For our control, the same amount of solvent was prepared without herbicide. A completely randomized design of dishes was arranged with three replications. All tissues were incubated in a growth chamber at 25°C under darkness for 12 h, then exposed for 24 h to continuous fluorescent white light at 250 μmol m^{-2} s^{-1} PPF. Electrolyte leakage into the bathing medium was determined by using a

conductivity meter (Model 1481-61; Cole-Parmer Instrument Co., Vernon Hills, IL, USA). After accounting for differences in the background electrical conductivity (EC) of our treatment solutions, the results were expressed as the relative change in EC upon exposure to light.

Lipid Peroxidation

Lipid peroxidation was estimated by evaluating the levels of malondialdehyde (MDA) production, using a slight modification of the thiobarbituric acid (TBA) method described by Buege and Aust (1978). At 24 h after herbicide treatment (same as described above for electrolyte leakage), the tissues were exposed to 250 μmol m^{-2} s^{-1} PPF at 25°C following 12 h of dark incubation, then separated from the bathing medium. The incubated tissues (100 mg) were homogenized with a mortar and pestle in 5 mL of 0.5% (v/v) TBA solution in 20% (v/v) trichloroacetic acid. The homogenate was centrifuged at 20,000*g* for 15 min and the supernatant was heated in a boiling water bath for 25 min, then cooled in an ice bath. This supernatant was centrifuged at 20,000*g* for 15 min, and the purified supernatant was used for spectrophotometric determination of MDA. Absorbance at 532 nm was recorded and corrected for nonspecific absorbance at 600 nm. MDA concentrations were calculated using an extinction coefficient of 156 mM^{-1} cm^{-1} and the following formula: MDA (μmol g^{-1} fresh weight) = $[(A_{532} - A_{600})/156] \times 10^3 \times$ dilution factor (Zhanyuan and Bramlage, 1992). The MDA concentrations from both the leaf tissue and the bathing medium were pooled and expressed as total MDA produced in terms of fresh biomass (Choi et al., 1996).

Protoporphyrinogen Oxidase Activity and Protoporphyrin IX Determination

Seedlings at the three-leaf stage were treated with (590 g ai ha^{-1}), oxyfluorfen (50 g ai ha^{-1}), carfentrazone-ethyl (850 g ai ha^{-1}), or oxadiazon (1270 g ai ha^{-1}). The application rates were those amounts that caused 50% biomass reduction in whole-plant bioassays. Leaves were harvested at 2 DAT, and PROTOX activity was determined using the method suggested by Lermontova and Grimm (2000). The substrate, Protogen IX, was prepared by chemical reduction of Proto IX with a sodium mercury amalgam (Sigma-Aldrich). The enzyme reaction was set up, incubated at 30°C for 5 min, and stopped by adding ice-cold methanol:dimethyl sulphoxide (8:2). Proto IX was separated via high-performance liquid chromatography (HPLC), using a Novapak C_{18} column (4-μm particle size, 4.6 × 250 mm; Waters Chromatography, Milford, MA, USA) 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 with a fluorescence detector (474, Waters) at excitation and emission wavelengths of 400 and 630 nm, respectively. To measure the Proto IX content, plant tissue (200 mg) was ground in 1 mL of methanol:acetone:0.1 N NaOH (9:10:1), and the homogenate was centrifuged at 10,000*g* for 10 min to remove cell debris. Proto IX was analyzed directly by HPLC (as described above), then identified and quantified using authentic standards.

Scanning Electron Microscopy (SEM)

Three-leaf-stage seedlings were treated with oxyfluorfen (50 g ai ha^{-1}). At 2 DAT, the leaves were harvested and fixed overnight at 4°C with 2% glutaraldehyde and 2% paraformaldehyde in 50 mM cacodylate buffer (pH 7.2). They were then washed three times with 50 mM cacodylate buffer (pH 7.2) and treated with 1% OsO_4 in 50 mM cacodylate buffer (pH 7.2), prior to washing three times with cacodylate buffer (pH 7.2). The prepared tissues were dehydrated in a solvent series of 50, 70, 90, 95, and 100% ethanol for 5 min each, then freeze-dried. For SEM, the dried tissues were coated with gold particles and examined at a magnification of 1,000 X under a scanning electron microscope (JSM 5410LV; JEOL Ltd, Tokyo, Japan).

Table 1. *Myxococcus xanthus* PROTOX transgene segregation pattern as determined by hygromycin-resistance test, and yield and yield components of the wild-type and transgenic rice lines in the T_3 generation. Disks were collected from transgenic leaves and incubated in water containing hygromycin (50 μg mL^{-1}).

Line[a]	Hygromycin resistance	Yield component					
	Fixing rates[b] (%)	Panicles per plant	Spikelets per panicle	Ripened grain (%)	1,000 grain weight (g)	Yield (kg ha^{-1})	Yield index[c]
WT	0	10.5b	75.0a	90.6a	28.4a	4,980a	100
TC	-	11.7b	71.7a	90.2a	27.3a	5,050a	101
M1	87	13.0a	64.6b	89.7a	26.8a	4,970a	100
M2	93	14.0a	57.8c	88.6a	27.9a	5,000a	100
M3	93	12.8a	55.7c	87.8a	28.0a	4,460b	89
M4	100	13.7a	62.4b	90.1a	25.9b	4,920a	99
M5	53	11.6b	60.7b	89.5a	27.1a	4,300b	86
M6	23	11.0b	67.7b	90.0a	24.8b	4,060b	82
M7	70	12.6a	60.7b	88.8a	26.6a	4,480b	90
M8	70	13.0a	67.7b	89.6a	26.1a	5,080a	102

[a]WT, wild-type rice plants; TC, herbicide-susceptible, transgenic control; M1, M2, M3, M4, M5, M6, M7, and M8, herbicide-resistant, transgenic rice plants.
[b]Fixing rates (%) were calculated relative to the number of individuals out of 30 plants per line.
[c]Yield index was calculated relative to the yield of the wild-type rice plants.

Transmission Electron Microscopy (TEM)

For transmission electron microscopy, three-leaf-stage seedlings were treated with oxyfluorfen (50 g ai ha^{-1}). At 36 and 48 DAT, leaves were harvested and prepared for examination via TEM. Small pieces were dehydrated in acetone and embedded in Spurr's low viscosity resin (Spurr, 1969). Ultrathin sections were then cut with a diamond knife on an RMC MTX ultramicrotome (Lee, 2006). The sections were stained with 1% potassium permanganate (KMnO4) (prepared in 0.1% sodium citrate) and examined under a transmission electron microscope (JEOL 1010; JEOL Ltd, Tokyo, Japan).

RESULTS AND DISCUSSION

Hygromycin Resistance and Grain Yield of Transgenic Rice Lines

At 6 days after treatment (DAT), tissues from hygromycin-susceptible rice plants were completely discolored (brown) while those from resistant plants were only slightly discolored or else retained their normal green color (data not shown). This confirmed hygromycin resistance in the transgenic rice. Line M4 was completely resistant (Table 1), indicating that the *Myxococcus xanthus* PROTOX gene was stably expressed at T_3. The other lines -- M1 to M3, and M5 to M8 -- showed only partial resistance.

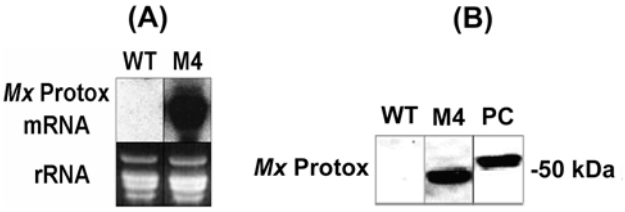

Figure 1. Transgene expression from *Myxococcus xanthus* PROTOX-overexpressing transgenic rice plants. **A,** Total RNA (10 μg) was blotted onto nylon membrane. **B**, Immunoblot of *M. xanthus* PROTOX protein. Equal amounts of protein (20 μg) from wild-type and transgenic rice were separated via SDS-PAGE, electro-transferred onto PVDF membranes, and incubated with primary antibodies raised against synthetic PROTOX peptides corresponded to C-terminal 19 amino acids of *M. xanthus* PROTOX. PC, 50 ng of partially purified recombinant *M. xanthus* PROTOX with C-terminal hexahistidine tag; WT, wild-type rice plants; M4, T_4 transgenic rice plants.

Grain yields from transgenic Lines M1, M2, M4, and M8 were similar to those from the wild type (WT) and the transgenic control (TC), while yields from Lines M3, M5, M6, and M7 were 10 to 18% lower than from the WT. Such reductions were mainly caused by a fewer number of spikelets per panicle and lower 1000-grain weights. Because Line M4 had a yield similar to the WT, it was selected for further study.

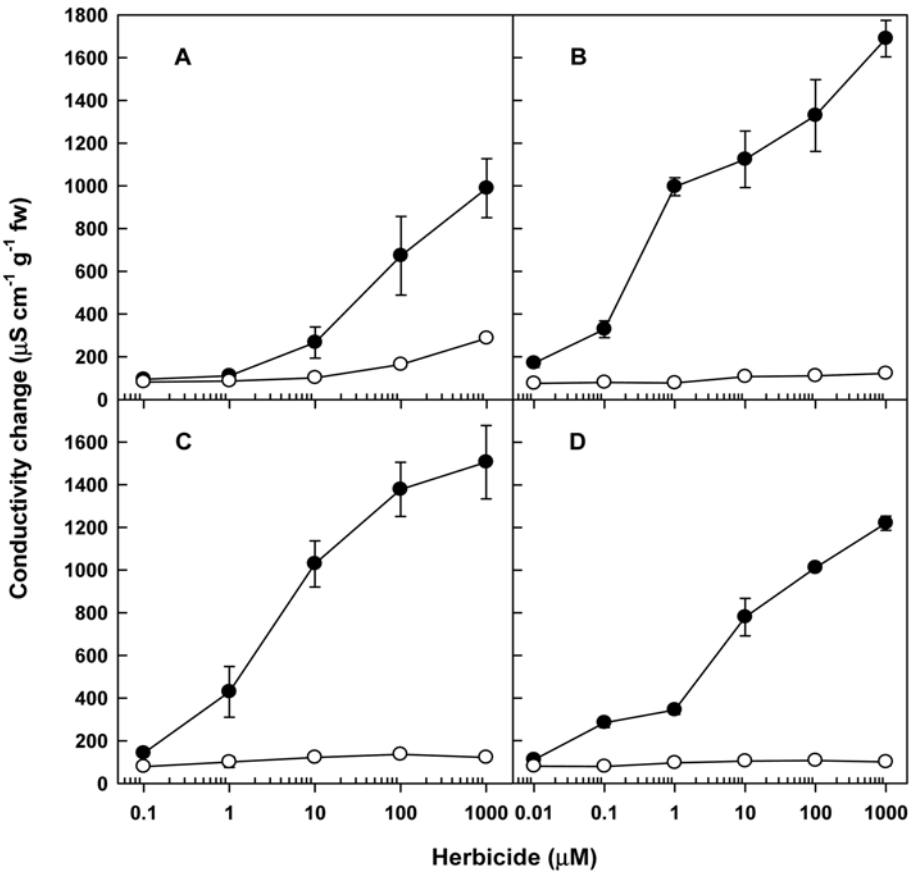

Figure 2. Effects of acifluorfen (**A**), oxyfluorfen (**B**), carfentrazone-ethyl (**C**), and oxadiazon (**D**) on electrolyte leakage from leaf squares of wild-type (WT, ●) and transgenic (M4, ○) rice plants. Tissues were exposed to continuous light at 250 μmol m^{-2} s^{-1} PPF at 25°C for 24 h following 12-h dark incubation. Each data point is mean ± SE of three replications. In some cases, error bar is obscured by symbol.

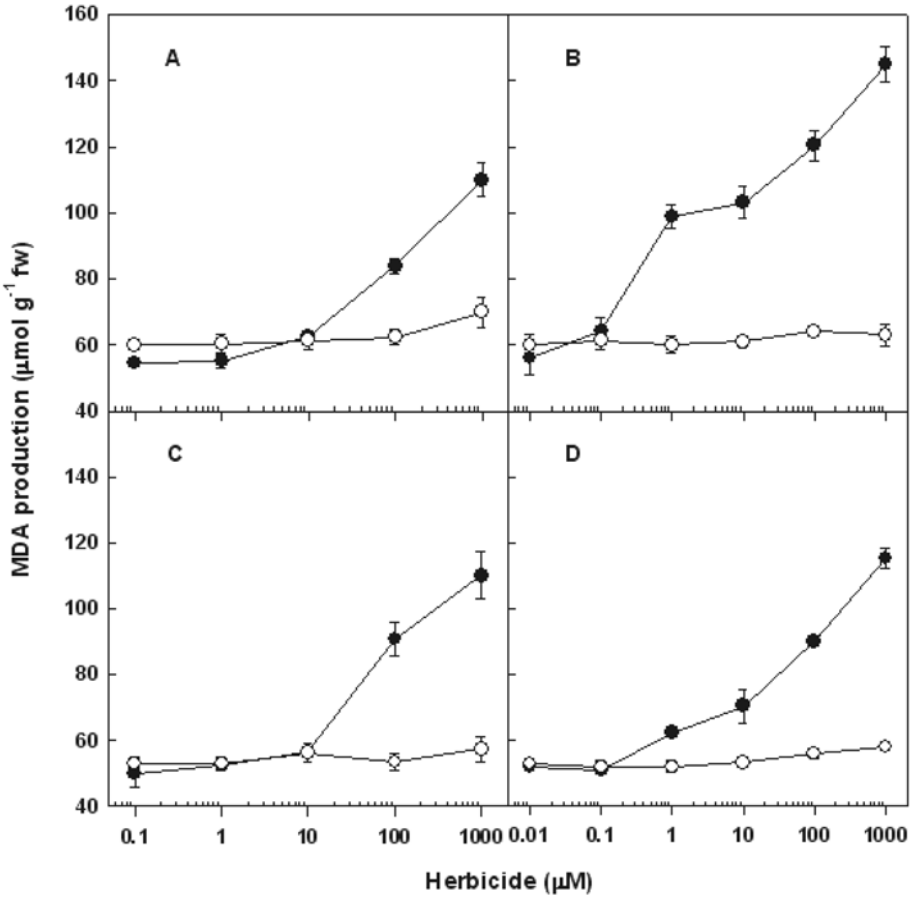

Figure 3. Effects of acifluorfen (**A**), oxyfluorfen (**B**), carfentrazone-ethyl (**C**), and oxadiazon (**D**) on malondialdehyde (MDA) production from leaf squares of wild-type (WT, ●) and transgenic (M4, ○) rice plants. Tissues were exposed to continuous light at 250 μmol m^{-2} s^{-1} PPF at 25°C for 24 h following 12-h dark incubation. Each data point is mean ± SE of three replications. In some cases, error bar is obscured by symbol.

Expression of *M. xanthus* PROTOX in Transgenic Rice Line M4

To determine whether M4 produces *M. xanthus* PROTOX mRNA, northern blot analysis was performed (Fig. 1a). WT mRNA produced no hybridization signal whereas *M. xanthus* PROTOX mRNA was expressed abundantly in the total RNA from M4 leaf tissues, indicating stable expression in the T_4 plants. Production of PROTOX protein was verified by specific antibody cross-reactivity with a protein having a predicted molecular mass of 50 kD in M4; no such protein was detected in the WT (Fig. 1b).

Physiological Responses

All four herbicides tested here -- acifluorfen, oxyfluorfen, carfentrazone-ethyl, and oxadiazon -- caused considerable cellular leakage from the treated leaves of WT plants exposed to light after 12 h of incubation under darkness (Fig. 2). The magnitude of this leakage was in proportion to herbicide concentration. In contrast, transgenic rice did not exhibit leakage when treated with concentrations of up to 1000 μM oxyfluorfen, carfentrazone-ethyl, and oxadiazon. Although transgenic plants did show some cellular leakage when exposed to 100 to 1000 μM acifluorfen, the magnitude was much lower than from the WT.

Similar results were observed for lipid peroxidation. MDA levels in WT rice increased at greater concentrations of PROTOX-inhibitor herbicides, but not in the transgenics (Fig. 3). Higher MDA levels may have been due to the accumulation of Proto IX in the cytoplasm, which subsequently generates AOS with light activation (Jacobs et al., 1991; Jung et al., 2004). Our results demonstrated that transgenic rice plants expressing the *M. xanthus* PROTOX gene are resistant to the PROTOX inhibitors used in this study.

PROTOX Activity and Proto IX Accumulation

PROTOX has been identified as a molecular target of PROTOX-inhibiting herbicides. Although the primary target is chloroplastic PROTOX, mitochondrial PROTOX is also important to the accumulation of Proto IX and to maintaining the flow of tetrapyrrole precursors for heme and chlorophyll biosynthesis (Watanabe et al., 1998). Thus, *M. xanthus* PROTOX is dually targeted into both chloroplasts and mitochondria to achieve a high level of herbicide resistance.

Although we assayed total PROTOX content for the combined fractions of crude chloroplasts and mitochondria, activity in untreated transgenic rice was 17-fold higher than that measured from the untreated WT (Fig. 4). Jung et al. (2004) also have found that chloroplastic and mitochondrial PROTOX activities are 5-fold and 12-fold higher in trans-

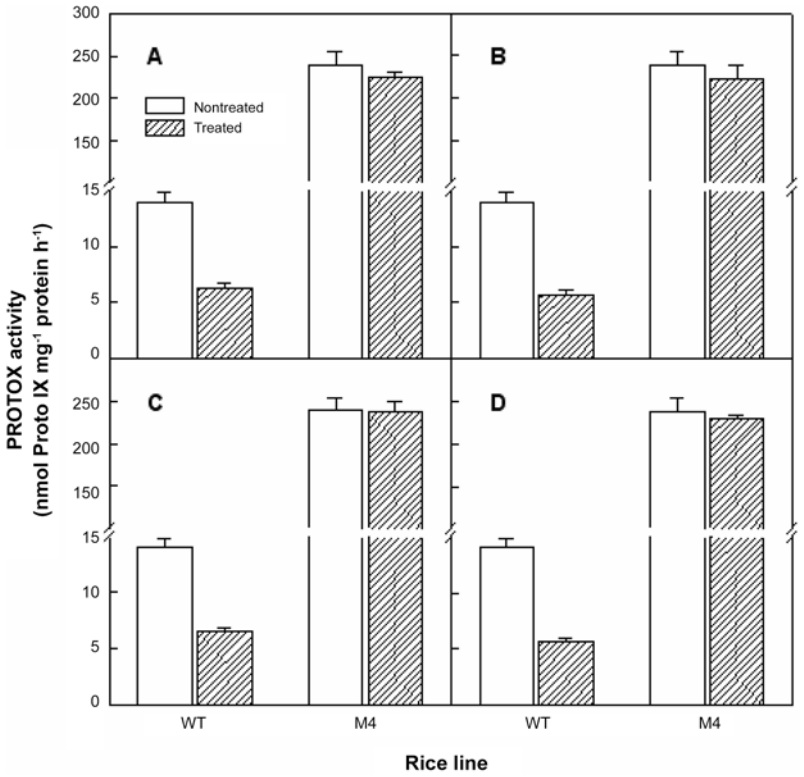

Figure 4. Changes in protoporphyrinogen oxidase (PROTOX) activity in wild-type (WT) and transgenic (M4) rice plants treated at three-leaf stage with acifluorfen (590 g ai ha^{-1}; **A**), oxyfluorfen (50 g ai ha^{-1}; **B**), carfentrazone-ethyl (850 g ai ha^{-1}; **C**), or oxadiazon (1270 g ai ha^{-1}; **D**). Total PROTOX activity was determined at 2 DAT. Each data point is mean ± SE of three replications. In some cases, error bar is obscured by symbol.

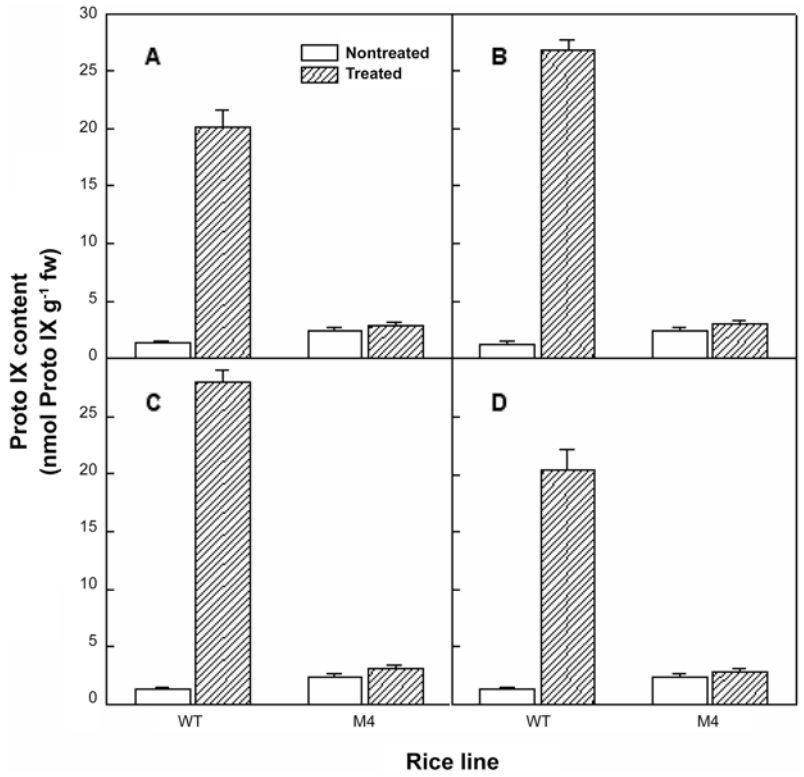

Figure 5. Changes in protoporphyrin (Proto IX) content in wild-type (WT) and transgenic (M4) rice plants treated at three-leaf stage with acifluorfen (590 g ai ha^{-1}; **A**), oxyfluorfen (50 g ai ha^{-1}; **B**), carfentrazone-ethyl (850 g ai ha^{-1}; **C**), or oxadiazon (1270 g ai ha^{-1}; **D**). Proto IX content was determined at 2 DAT. Each data point is mean ± SE of three replications. In some cases, error bar is obscured by symbol.

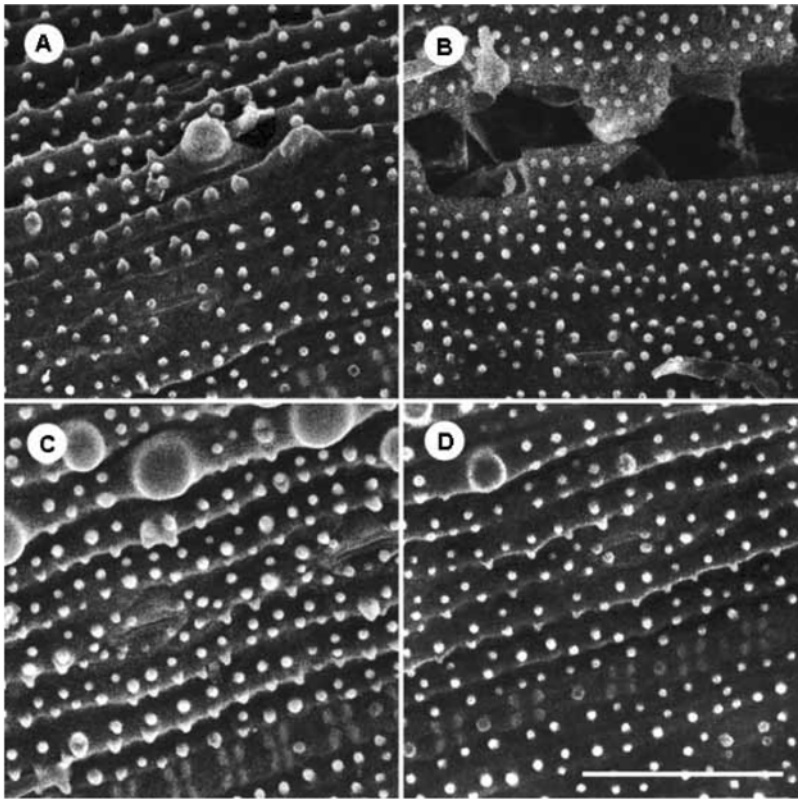

Figure 6. Scanning electron microscopy (SEM) of wild-type (**A**, untreated; **B**, treated) and transgenic (**C**, untreated; **D**, treated) rice leaves 48 h after selected three-leaf-stage plants were treated with oxyfluorfen (50 g ai ha^{-1}). Bar = 50 μm.

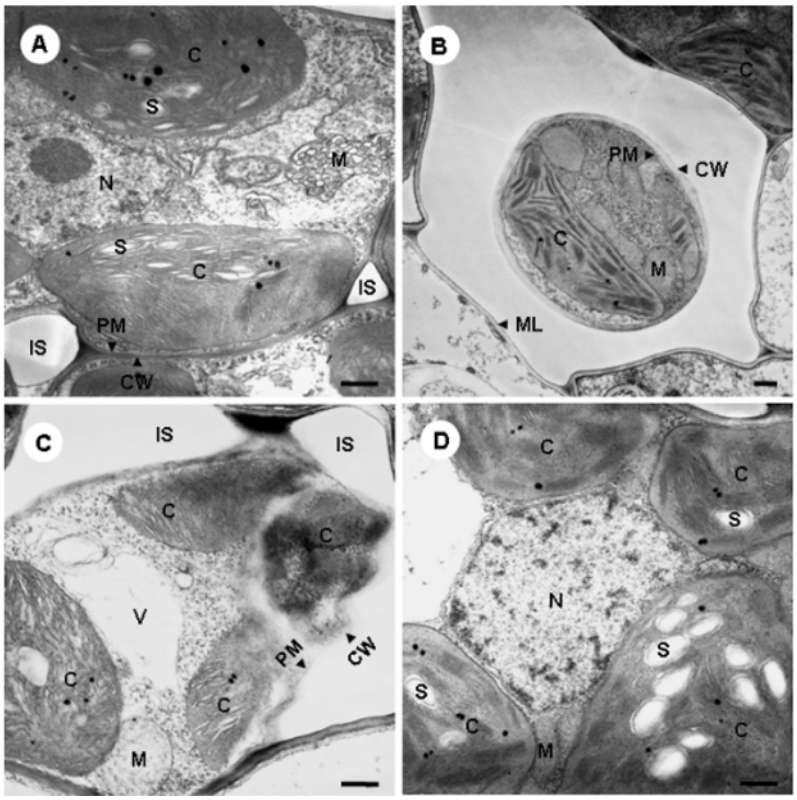

Figure 7. Ultrastructure of epidermal cells from leaves of untreated (**A**) and treated wild-type (**B and C**) and transgenic (**D**) rice at 36 h (**B**) or 48 h (**C, D**) after selected three-leaf-stage seedlings were treated with oxyfluorfen (50 g ai ha^{-1}). C, chloroplast; CW, cell wall; IS, intercellular space; M, mitochondria; ML, middle lamella; N, nucleus; PM, plasma membrane; S, starch grain; V, vacuole. Bars = 500 nm.

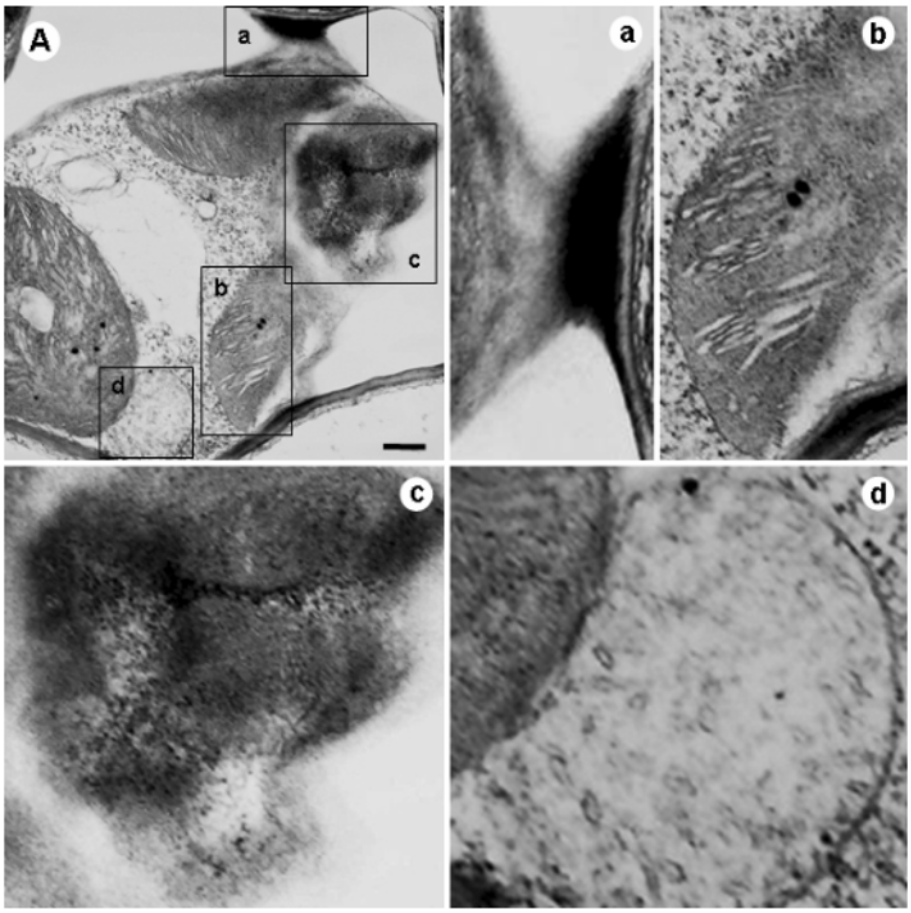

Figure 8. Disruption of ultrastructures in wild-type rice plants at 48 h after oxyfluorfen treatment (50 g ai ha^{-1}) (**A**). Close-up view of plasma membrane (**a**), chloroplast (**b**), destroyed chloroplasts (**c**), and mitochondria (**d**). Bar = 500 nm.

genic rice expressing *M. xanthus* PROTOX than in WT rice, respectively. It is believed that overexpression of a specific target enzyme generally can lead to herbicidal resistance in many crop plants. Here, overexpressed *M. xanthus* PROTOX in the chloroplasts and mitochondria from transgenic rice appeared to be correlated with resistance to acifluorfen, oxyfluorfen, carfentrazone-ethyl, and oxadiazon (Fig. 4). There is also evidence that overexpression of either chloroplastic or mitochondrial PROTOX enhances resistance to PROTOX-inhibiting herbicides (Lermontova and Grimm, 2000; Watanabe et al., 1998). Furthermore, transgenic maize over-expressing the *Arabidopsis* PROTOX mutant gene shows a 1,000-fold higher degree of resistance to the PROTOX-inhibiting herbicide butafenacil (Li et al., 2003). Therefore, overexpression of the PROTOX gene neutralizes herbicidal action, preventing the accumulation of the substrate Protogen IX and, consequently, eliminating the light-dependent phytotoxity of PROTOX-inhibiting herbicides (Lermontova and Grimm, 2000; Watanabe et al., 1998). In our WT rice, PROTOX activities were inhibited 55%, 59%, 53%, and 60% by acifluorfen, oxyfluorfen, carfentrazone-ethyl, and oxadiazon, respectively, compared with the untreated control. Enzyme assays showed that PROTOX activities in transgenic rice were similar to those in the untreated control (Fig. 4).

The Proto IX content from untreated transgenic rice was about 2-fold higher than from the untreated WT (Fig. 5). Its accumulations in the latter were increased 15-, 20-, 21-, and 16-fold by treatment with acifluorfen, oxyfluorfen, carfentrazone-ethyl, and oxadiazon, respectively, compared with no significant accumulation in the transgenics. Even with herbicide treatment, elevated PROTOX activity in the latter type likely ensured normal metabolic flow in the porphyrin pathway and prevented the accumulation of photosensitizing Proto IX, thereby minimizing cellular leakage and lipid peroxidation (Figs. 2, 3). This response by transgenic rice is similar to that of an oxyfluorfen-resistant soybean cell line that over-expresses mitochondrial PROTOX (Warabi et al., 2001). A low level of Proto IX after treatment with a PROTOX-inhibiting herbicide also has been reported with tobacco mutant cell cultures (Ichinose et al., 1995). Thus, we may conclude that herbicide resistance by our transgenic rice expressing the *M. xanthus* PROTOX gene was a result of reduced Proto IX accumulation in the cytoplasm because of the increased expression and activity of PROTOX.

Microscopic analyses

The epicuticular wax on WT plants, but not on the transgenics, was damaged 48 h after oxyfluorfen treatment (Fig. 6). The usual features of normal chloroplasts were apparent in the cells of untreated WT rice (Fig. 7A) and numerous stacked grana were located in the thylakoids. A large portion

of the chloroplast volume was filled with starch, the remainder containing healthy mitochondria. The outer membrane was clearly defined. Cell ultrastructure did not differ significantly between the untreated WT and the treated transgenic rice (Fig. 7D). However, the former exhibited typical plasmolysis at 36 h after treatment with oxyfluorfen (Fig. 7B). Furthermore, the WT cells lost their reticulate network in the thylakoid membranes at 48 h post-treatment (Fig. 7C), with remnants of the cell membrane scattered throughout the stroma. This indicated a disintegration of the chloroplast and thylakoid membranes. The outer chloroplast membrane also was almost totally ruptured in several locations (Figs. 7C; 8b, c), and chloroplast contents had spilled into the cytoplasm (Figs. 7C; 8b). Structural damage was especially severe in the plasma membrane following oxyfluorfen treatment (Figs. 7C; 8a). The insides of the mitochondria were nearly empty and electron density was significantly reduced (Figs. 7C; 8d).

In conclusion, our research results suggest that rice plants may acquire resistance to PROTOX-inhibiting herbicides by over-expressing both mitochondrial and chloroplastic PROTOX. Proto IX does not accumulate in transgenic plants because of the overproduction and increased activity of this chloroplastic and mitochondrial PROTOX.

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