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Expression of a *Bacillus subtilis* protoporphyrinogen oxidase gene in rice plants reduces sensitivity to peroxidizing herbicides

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Abstract

Protoporphyrinogen oxidase (Protox) in the porphyrin pathway is the target site of the peroxidizing herbicides such as carfentrazone-ethyl and oxyfluorfen. In an attempt to develop herbicide-resistant plants, transgenic rice plants were generated *via* expression of herbicide-insensitive *Bacillus subtilis* Protox gene fused to the transit sequence for targeting to the plastid using *Agrobacterium*-mediated gene transformation. Homozygous transgenic rice lines of T_3 generation selected by hygromycin resistance test were examined if they are resistant to the herbicides carfentrazone-ethyl and oxyfluorfen. The homozygous transgenic lines had single copy insertion of *B. subtilis* Protox gene into their genomes and express its mRNA. Compared to wild-type rice, the transgenic lines were less susceptible to the herbicides when examined with respect to growth, electrolyte leakage, chlorophyll loss and lipid peroxidation. The *in vitro* Protox activities in transgenic lines were about 56 % higher than those in wild-type rice. With 10 μM concentration of the herbicides in the enzyme assays, Protox activities in transgenic lines were similar to those in non-inhibited wild-type rice. Less amount of protoporphyrin IX was accumulated in transgenic lines than in wild-type rice upon the treatment of the herbicides at 10 μM concentration. Our results indicated that expression of *B. subtilis* Protox gene was stably transmitted into T_3 rice plants and reduced their sensitivity to carfentrazone-ethyl and oxyfluorfen.

Additional key words: gene transformation, herbicide resistance, lipid peroxidation, porphyrin pathway.

Introduction

Several herbicides that cause rapid bleaching and desiccation of green plant tissues have been found to inhibit protoporphyrinogen oxidase (Protox), the last common enzyme in the porphyrin pathway leading to the formation of both heme and chlorophylls (Chl) (Beale and Weinstein 1990, Scalla and Matringe 1994). The biochemical basis for the mechanism of action of the herbicides is the competitive inhibition of plastid Protox, whereby protoporphyrinogen IX (Protogen), the substrate of the enzyme, is accumulated in the plastid envelope, diffused into the cytosol and transported to the plasma membrane, where it is rapidly oxidized to protoporphyrin IX (Proto) by a herbicide-insensitive peroxidase-like enzyme (Lee and Duke 1994). Resulting Proto generates singlet oxygen and provokes membrane lipid peroxidation leading to cellular death via a light-dependent mechanism (Scalla and Matringe 1994).

No resistant plants to the peroxidizing herbicides in nature have been reported. The reason for this might be the fact that the herbicides exert their effect very rapidly and

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Abbreviations: Chl - chlorophyll(s); EC - electrical conductivity; MDA - malondialdehyde; PAR - photosynthetically active radiation; Proto - protoporphyrin IX; Protogen - protoporphyrinogen IX; Protox - protoporphyrinogen oxidase.

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thus selection pressure on plants is short-lived (Duke *et al.* 1997). Furthermore, plants having the altered Protox to the herbicides cannot survive due to its low affinity for Protogen. Since herbicide-resistant weeds are unlikely to arise rapidly in such crops and the herbicides are toxicologically and environmentally safe, the peroxidizing herbicides are suitable for development of herbicide--resistant crops.

All eukaryotic Protox that have been characterized so far are inhibited by the herbicides, but the Protox from *Bacillus subtilis* is known to be insensitive to the herbicides (Jacobs *et al.* 1990, Dailey *et al.* 1994). Furthermore, the Protox gene of *B. subtilis* has recently been cloned and expressed in *Escherichia coli* (Dailey *et al.* 1994), tobacco (Choi *et al.* 1998) and rice (Lee *et al.* 2000). In fact, the transgenic tobacco and rice expressing *B. subtilis* Protox under cauliflower mosaic virus 35S and ubiquitin promotor, respectively, were found to be resistant to oxyfluorfen in T_0 generation (Choi *et al.* 1998, Lee *et al.* 2000). The transgenic rice plants were generated via expression of *B. subtilis* gene in the cytosol or in the plastid using *Agrobacterium*-mediated gene transformation (Lee *et al.* 2000). The plastidal transgenic lines exhibited much higher expression of the transgene mRNA and higher herbicide resistance than the cytosolic transgenic lines.

In the present study, the expression of *B. subtilis* Protox gene in the plastidal transgenic rice lines was examined to determine if it is stably transmitted into next generation. Growth and physiological responses of homozygous transgenic rice lines of T_3 generation were compared with those of wild-type rice to examine their sensitivity to the peroxidizing herbicides carfentrazone-ethyl and oxyfluorfen.

Materials and methods

Transformation and regeneration: *Bacillus subtillis* Protox gene construct (Lee *et al.* 2000) was prepared using conventional molecular biology techniques. For expressing the *B. subtilis* Protox in the plastid, the transit sequence (186 nt) derived from a tobacco Protox gene was fused in frame in front of the *B. subtilis* gene. The procedures for *Agrobacterium*-mediated transformation and subsequent regeneration were described previously (Lee *et al.* 2000). Briefly, the calli induced from scutellum of rice (*Oryza sativa* cv. Nackdong) seeds were co-cultured with *A. tumefaciens* LBA4404 harboring the gene construct for 2 - 3 d in darkness at 25 °C. The co-cultivated calli were transferred to N6 media containing 250 mg dm^{-3} cefotaxime and 50 mg dm^{-3} hygromycin. After selection for 3 - 4 weeks, the calli were transferred to a regeneration media for shoot and root development. After the roots had sufficiently developed, the transgenic plants were transferred to a greenhouse and grown to maturity.

Plants: Homozygous transgenic rice lines of T_3 generation were screened by hygromycin resistance test (Wang and Waterhouse 1997). Two 4-mm diameter leaf discs each from transgenic rice lines at 3- to 4-leaf stage were placed with 10 replications in a 24-well culture plate (*Nalge Nunc International*, Rochester, NY, USA) containing 0.1 mg cm^{-3} hygromycin and then incubated at 25 °C for 3 d under light condition at 100 μmol m^{-2} s^{-1} photosynthetically active radiation (PAR). When all leaf discs from transgenic line remained green, the transgene was assumed to be presented as a dominant homozygous form, since the transgene was closely linked to the hygromycin-resistant gene (Lee *et al.* 2000). Among the transgenic lines without showing the segregation of hygromycin-resistant trait, two homozygous transgenic lines, T-32 and T-72, were used for this study.

DNA and RNA blot analysis: Genomic DNA was isolated according to standard methods (Ausubel *et al.* 1987). Genomic DNA (5 μg) was digested by a *Hin*dIII restriction enzyme, size-fractionated by electrophoresis in 0.8 % agarose gels, blotted to nylon membranes (*Nylon 66 plus, Pharmacia*, Piscataway, NJ, USA) and hybridized with the *B. subtilis* Protox gene. Total RNA (10 μg) was isolated from leaves of T_3 transgenic or wild-type rice plants using *TRI* reagent (*Sigma Chemical Co.*, St. Louis, MO, USA) and fractionated on 1 % agarose gel containing formaldehyde using 20 mM 3-(*N*-morpholino)propanesulfuric acid as a running buffer. The gel was blotted to a nylon membrane and hybridized with the full length of the *B. subtillis* Protox gene. RNA samples were stained by ethidium bromide prior to blotting.

Herbicides: Technical-grade herbicides (> 99 %) of carfentrazone-ethyl [ethyl 2-chloro-3-(2-chloro-4-fluoro-5-(4-(difluoromethyl)-4,5-dihydro-3-methyl-5-oxo-1*H*-1,2,4-triazol-1-yl)phenyl)propionate] and oxyfluorfen [2-chloro-1-(3-ethoxy-4-nitrophenoxy)-4-(trifluoromethyl)benzene] were generously provided by *FMC Korea Co., Ltd.* (Seoul, Korea) and *Rohm and Haas Co., Ltd.* (Seoul, Korea), respectively.

Whole plant response: Seeds of wild-type and T_3 transgenic rice lines were soaked in distilled water for 4 d at 25 °C. The seeds were sowed in plastic pots (14 × 9 × 9 cm) filled with commercial soil substrate, and grown in a greenhouse at 30 ± 5/20 ± 5 °C, day/night temperature with an approximately 14 h photoperiod. Carfentrazone-ethyl was treated at 1.25, 2.5 and 5.0 mg(active ingredient) m^{-2} under a submerged condition with 3 cm water depth, whereas oxyfluorfen was sprayed to the soil at 1.8, 7.2 and 28.8 mg(active ingredient) m^{-2} under dry conditions 3 d after seeding. Shoot fresh mass was determined 10 d after the treatment. All treatments were triplicated.

Electrolyte leakage: The leaf blades of 4-week-old rice plants were treated with carfentrazone-ethyl and oxyfluorfen as mentioned before (Lee *et al*. 1995) by cutting 4-mm leaf squares (100 mg) with a razor blade, and then placing them in a 6-cm diameter polystyrene Petri dish containing 5 cm^3 of 1 % sucrose, 1 mM 2-(*N*-morpholino)ethanesulfuric acid (pH 6.5) with or without the herbicide dissolved in acetone. The control contained the same amount of the solvent without the herbicide. The final concentration of acetone in all dishes was 1 % (v/v). The tissues were incubated in a growth chamber at 25 °C in darkness for 12 h and then exposed to continuous fluorescent white light at 250 μmol m^{-2} s^{-1} PAR for 24 h. Electrolyte leakage into the bathing medium was determined periodically using a conductivity meter (*Model 1481-61, Cole-Parmer Instrument Co*., Vernon Hills, IL, USA). Because of differences in background electrical conductivity (EC) of different treatment solutions, results were expressed as changes in EC upon exposure to light. All treatments for each measurement were triplicated.

Chl determination: Chl content was determined after 24 h of exposure to 250 μmol m^{-2} s^{-1} PAR at 25 °C following 12 h dark incubation. Chl was extracted and assayed according to the procedure of Hiscox and Israelstam (1979). The tissues from the dishes were soaked for 48 h in darkness in 10 cm^3 of dimethyl sulfoxide at room temperature. The total Chl content in extracts was determined using a spectrophotometer (*Model UV-2401PC, Shimadzu Corp*., Kyoto, Japan).

Lipid peroxidation: Lipid peroxidation was estimated by malondialdehyde (MDA) production using a modification of the thiobarbituric acid method (Buege and Aust 1978). After 24 h of exposure of the herbicide-treated tissues to 250 μmol m^{-2} s^{-1} PAR at 25 °C following 12 h dark incubation, they were separated from the bathing medium. The MDA concentrations on a fresh mass basis from both fractions of the tissues and the bathing medium were pooled and then regarded as a total MDA produced by the tissues (Choi *et al*. 1996)

Protox assay: From the leaf material of 2-week-old rice plants grown in a greenhouse, plastids were isolated by the method of Lermontova and Grimm (2000). Protox in the plastid suspension was assayed by measuring formation of Proto from Protogen under strictly reducing condition with the method of Jung *et al*. (2003). The amount of Proto formed in the heat-denatured sample was subtracted from the Proto formed in the native sample (Guo *et al*. 1991). Inhibition of Protox in the plastid suspension was tested by applying a final concentration of 10 μM carfentrazone -ethyl or oxyfluorfen to the assay. Proto was separated by 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 min cm^{-3}. Proto was eluted with a solvent system of 0.1 M ammonium phosphate (pH 5.8) and methanol. The eluate was monitored with a fluorescence detector (*Model 474, Waters*) at excitation and emission wavelengths setting 405 and 625 nm, respectively (Kruse *et al*. 1995).

Proto determination: All extractions for HPLC determination of Proto were made under a dim, green light source after 12 h of incubation in darkness at 25 °C. Leaf samples of 100 mg were homogenized in 1 cm^3 of methanol:acetone:0.1 M NaOH (9:10:1, v/v/v) and the homogenate was centrifuged at 10 000 *g* for 10 min. The resulting supernatant was filtered through a 0.2 μm nylon syringe filter and then stored in light-tight, aluminum foil-wrapped, glass vials at -20 °C until analysis by HPLC. Proto was directly analyzed by HPLC as described above.

Results

DNA and RNA blot analysis: To assess the integration of *B. subtilis* Protox gene into rice genome of the T_3 transgenic lines, genomic DNA was extracted separately from the transgenic lines, digested with *Hin*dIII and hybridized with ^{32}P-labeled *B. subtilis* Protox gene. Due to the absence of *Hin*dIII site within the probed transgene, the number of hybridized bands directly corresponded to the copy number of transgene in genome of transgenic lines. The transgenic lines showed a single band about 5 kb in size (Fig. 1*A*), suggesting that they had single copy insertion in their genomes.

Total RNA was isolated from leaves of transgenic rice lines and examined by Northern blot with a probe that detects *B. subtilis* Protox mRNA. No hybridization signal was detected in RNA from wild-type rice plants (Fig. 1*B*). In contrast, *B. subtilis* Protox mRNA was expressed abundantly in both homozygous transgenic lines, showing that the expression of *B. subtilis* Protox gene was stably transmitted into T_3 rice plants.

Whole plant responses: To compare the growth of wild-type and transgenic rice lines, shoot fresh mass was determined 10 d after carfentrazone-ethyl and oxyfluorfen treatments. There were no significant differences in shoot fresh mass of the control samples between wild-type and transgenic line 10 d after seeding in either submerged conditions with 3 cm water depth or directing seeding on dry conditions (data not shown). The transgenic lines appeared to have normal phenotypes and none of them displayed bleached or necrotic leaf lesions as a result of the transgene expression. Carfentrazone-ethyl at a rate of 1.25 or 2.5 g(active ingredient) m^{-2} effectively inhibited growth of wild-type rice, but less inhibition was found in transgenic lines (Fig. 2). Similar results were obtained with oxyfluorfen treatment. Photodynamic injury symptoms such as chlorosis and leaf rolling caused by the treatment of carfentrazone-ethyl and oxyfluorfen at 1.25 - 2.5 and 1.8 - 14.4 g(active ingredient) m^{-2}, respectively, were obvious in wild-type, but not observed in transgenic lines (data not shown). These results indicate that transgenic rice lines are resistant to the herbicides. Since the transgenic line of T-32 showed slightly higher resistance than T-72, the subsequent experiments were conducted with wild-type and T-32.

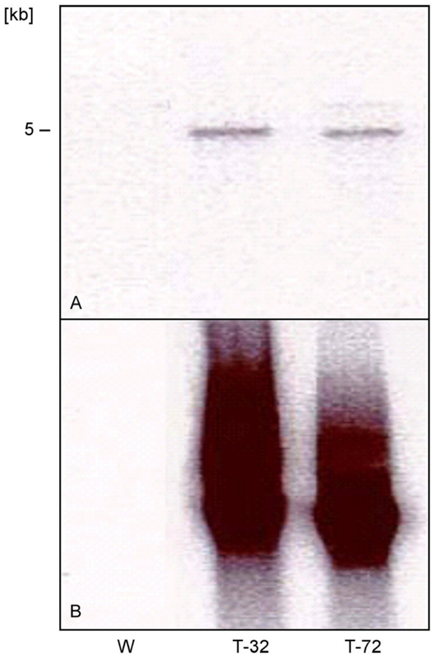

Fig. 1. Southern (*A*) and Northern blot analysis (*B*) of wild-type and transgenic rice plants. W - wild-type, T-32 and T-72 - transgenic lines.

Physiological responses: Carfentrazone-ethyl and oxyfluorfen caused considerable electrolyte leakage from the treated leaf squares of wild-type rice plants upon the exposure of light following 12 h incubation in darkness (Figs. 3*A*,*B*). EC increased depending on the concentration of the herbicides. However, EC was much lower in transgenic than in wild-type rice treated with varying concentrations of the herbicides (Figs. 3*A*,*B*).

Chl contents in wild-type and transgenic line in the untreated control were not different, indicating that *B. subtilis* Protox gene expression had no significant effect on Chl contents. Both carfentrazone-ethyl and oxyfluorfen effectively reduced Chl content in leaves from wild-type rice in a concentration-dependent manner, but little or no effects were observed in the treated leaves from the transgenic line (Figs. 3*C*,*D*).

Carfentrazone-ethyl and oxyfluorfen also caused significant lipid peroxidation in the treated leaves of wild-type rice (Figs. 3*E*,*F*). However, less lipid peroxidation was detected in transgenic line than in wild-type rice.

Protox activity and Proto accumulation: Protox activity was 56 % higher in the transgenic line than in wild-type (Fig. 4*A*), indicating that *B. subtilis* Protox gene produces an enzyme that is functionally active in transgenic rice line. The catalytic oxidation in wild-type rice was about 64.8 nmol(Proto) g^{-1} (protein) s^{-1}. Wild-type Protox activity was 58 and 70 % inhibited by the addition of 10 μM carfentrazone-ethyl and oxyfluorfen to the enzyme assay, respectively. With 10 μM carfentrazone-ethyl or oxyfluorfen in the enzyme assays, however, Protox activities in transgenic line were similar to those in non-inhibited wild-type extracts. Protox activities of transgenic line were 22 and 26 % less inhibited than those of wild-type by carfentrazone-ethyl and oxyfluorfen, respectively.

There was no significant Proto accumulation in control tissues of wild-type and transgenic line after 12 h in darkness (Fig. 4*B*). With 10 μM carfentrazone-ethyl or oxyfluorfen, however, Proto was accumulated in the treated tissues with lower amount in transgenic line than in wild-type.

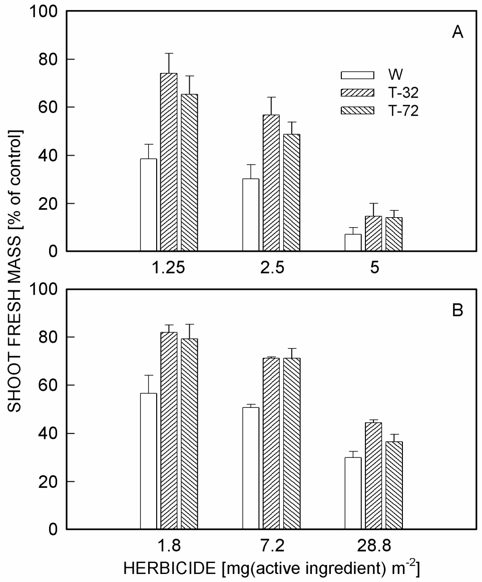

Fig. 2. Effect of carfentrazone-ethyl (*A*) and oxyfluorfen (*B*) on growth of wild-type and transgenic rice plants. W - wild-type, T-32 and T-72 - transgenic lines. Data represent the mean ± SE.

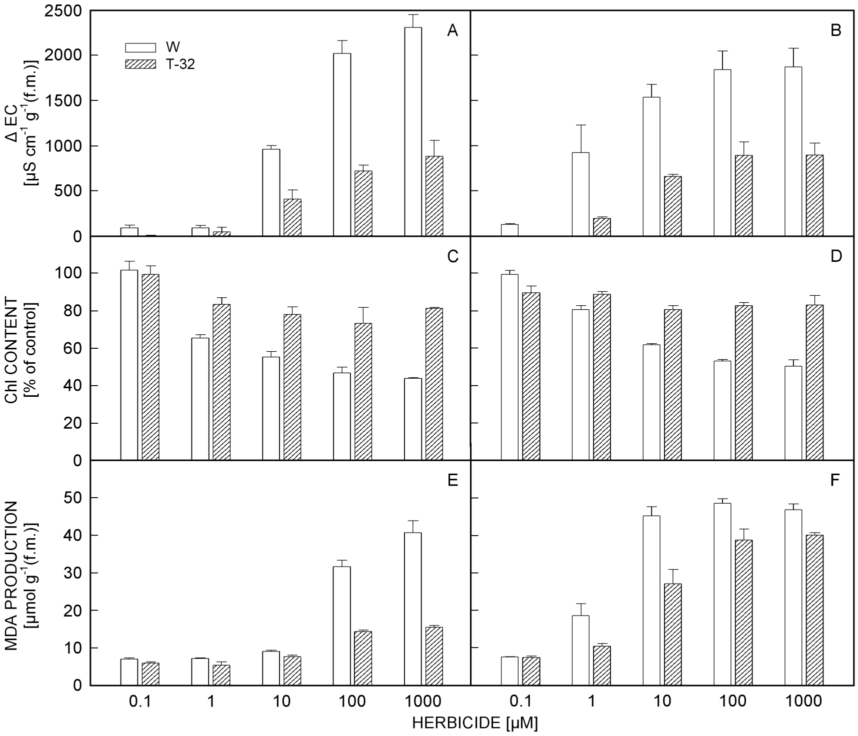

Fig. 3. Effect of carfentrazone-ethyl (*A*, *C*, *E*) and oxyfluorfen (*B*, *D*, *F*) on electrolyte leakage (*A*, *B*), Chl loss (*C*, *D*) and MDA production (*E*, *F*) from leaf squares of wild-type and transgenic rice plants. W - wild-type, T-32 - transgenic line. Values of electrolyte leakage and MDA production are differences between treated and control tissues. Chl content in control tissues was 1.1 mg g^{-1}(f.m.). Data represent the mean ± SE.

Discussion

Several attempts have been made for generating plants resistant to the peroxidizing herbicides. A S23142-resistant cell line of photomixotrophically cultured tobacco was selected after stepwise increasing of the concentration of the herbicide (Ichinose *et al*. 1995). Oxyfluorfen-resistant non-chlorophyllous soybean cells were selected which contained Protox that was 12-fold less sensitive to the herbicide than wild-type cells (Pornprom *et al*. 1994). However, whether this would translate into a significant increase in resistance in a plant regenerated from these cells was unknown. A point mutation, Val-389-Met, of Protox conferred S23142-resistance to the rs-3 mutant of *Chlamydomonas reinhardtii* (Randolph-Anderson *et. al*. 1998). Over-expression of *Arabidopsis* Protox gene in the plastid of transgenic tobacco compensated for acifluorfen effects by preventing the accumulation of Proto and thereby led to resistance to the herbicide (Lermontova and Grimm 2000).

We have generated the transgenic rice plants expressing the herbicide-insensitive *B. subtilis* Protox gene targeted to the plastid using *Agrobacterium*-mediated gene transformation (Lee *et al*. 2000, Jung *et al*. 2003). The homozygous transgenic rice lines of T_3 generation had single copy insertion of the transgene in their genome

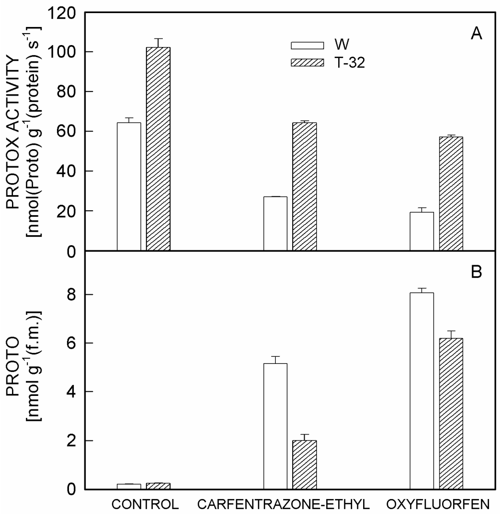

Fig. 4. Effect of carfentrazone-ethyl and oxyfluorfen on Protox activity (*A*) and Proto accumulation (*B*) in plastid suspensions from wild-type and transgenic rice plants. W - wild-type, T-32 - transgenic line. Protox activity was measured with or without 10 μM herbicides. Proto accumulation was determined after incubating the tissues with or without 10 μM herbicides in darkness at 25 °C for 12 h. Data represent the mean ± SE.

(Fig. 1*A*) and *B. subtilis* Protox mRNA was expressed abundantly in the transgenic lines (Fig. 1*B*). This result shows that the *B. subtilis* Protox gene was transmitted stably into T_3 rice plants and heterologous expression of Protox in rice was successful.

The transgenic lines were less sensitive to carfentrazone-ethyl and oxyfluorfen when examined with respect to growth (Fig. 2), cellular leakage (Figs. 3*A*,*B*), Chl loss (Figs. 3*C*,*D*) and lipid peroxidation (Figs. 3*E*,*F*). However, the responses to the herbicides were not exactly the same in all parameters tested. The reduced sensitivity might be attributed to the higher Protox activity in transgenic rice than in wild-type (Fig. 4*A*). Protox activity in transgenic rice could be the sum of activities from herbicide-sensitive endogenous Protox and herbicide--insensitive *B. subtilis* Protox. In the presence of herbicide, thus, the herbicide-insensitive Protox activity in transgenic rice would secure the normal metabolic flow in the porphyrin pathway and prevent the accumulation of photosensitizing Proto. Lesser amount of accumulated Proto in transgenic line (Fig. 4*B*) will account for the lesser herbicide effects on growth, cellular leakage, Chl loss and lipid peroxidation.

In the transgenic lines, *B. subtilis* Protox expressed in the plastid oxidizes Protogen to Proto regardless of the presence of the peroxidizing herbicides, presumably at a higher rate than the endogenous plastid Protox in the absence of the herbicides. Although the intermediates of the porphyrin pathway beyond Protogen will accumulate in the plastid membrane and act as a photosensitizer, this phenomenon might be overcome by feedback inhibition of δ-aminolevulinic acid synthesis, the committed step of the pathway, by heme and protochlorophyllide (Beale and Weinstein 1990). Thus, the transgenic lines will show reduced sensitivity to the peroxidizing herbicides and accomplish the normal porphyrin pathway. However, the localization of Protox gene expression in the transgenic lines must be determined precisely in the near future. Combination of resistance mechanisms might increase resistance level against peroxidizing herbicides. We intend to further explore the overexpression of plant Protox gene in combination with *B. subtilis* gene in rice with different cultivars.

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