Transgenic Rice Plants Expressing a *Bacillus subtilis* Protoporphyrinogen Oxidase Gene Are Resistant to Diphenyl Ether Herbicide Oxyfluorfen

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Protoporphyrinogen oxidase (Protox), the penultimate step enzyme of the branch point for the biosynthetic pathway of Chl and hemes, is the target site of action of diphenyl ether (DPE) herbicides. However, *Bacillus subtilis* Protox is known to be resistant to the herbicides. In order to develop the herbicide-resistant plants, the transgenic rice plants were generated via expression of *B. subtilis* Protox gene under ubiquitin promoter targeted to the cytoplasm or to the plastid using *Agrobacterium*-mediated gene transformation. The integration and expression of the transgene were investigated at T₀ generation by DNA and RNA blots. Most transgenic rice plants revealed one copy transgene insertion into the rice genome, but some with 3 copies. The expression levels of *B. subtilis* Protox mRNA appeared to correlate with the copy number. Furthermore, the plastidal transgenic lines exhibited much higher expression of the Protox mRNA than the cytoplasmic transgenic lines. The transgenic plants expressing the *B. subtilis* Protox gene at T₀ generation were found to be resistant to oxyfluorfen when judged by cellular damage with respect to cellular leakage, Chl loss, and lipid peroxidation. The transgenic rice plants targeted to the plastid exhibited higher resistance to the herbicide than the transgenic plants targeted to the cytoplasm. In addition, possible resistance mechanisms in the transgenic plants to DPE herbicides are discussed.

**Key words:** Diphenyl ether herbicides — Herbicide resistance — Oxyfluorfen — Protoporphyrinogen oxidase — Transgenic rice plants.

Protoporphyrinogen oxidase (Protox), which catalyzes the oxidation of protoporphyrinogen IX (Protogen IX) to protoporphyrin IX (Proto IX), is the penultimate step enzyme of the branch point for the biosynthetic pathway of Chl and hemes (Beale and Weinstein 1990). Numerous investigations, initially at the intact tissue level and more recently with cell-free preparations and purified enzymes, have revealed that Protox is inhibited by diphenyl ether (DPE) herbicides (Matringe et al. 1989, Duke et al. 1991, Lee et al. 1993, Scalla and Matringe 1994, Corrigall et al. 1998). The inhibition of Protox by the herbicides ultimately causes massive accumulation of Protogen IX, a photosensitizer, which produces singlet oxygen and provokes membrane lipid peroxidation leading to cellular death via a light-dependent mechanism (Duke et al. 1991, Scalla and Matringe 1994).

To date, a dozen of Protox genes have been cloned and characterized from *Escherichia coli*, yeast, human, and plants, each of which shares low amino acid identities among different organisms, but high homology between closely related families (Dailey et al. 1994, Dailey and Dailey 1996, Lermontova et al. 1997, Adomat and Böger 1999). All eukaryotic Protox that have been characterized so far are inhibited by DPE herbicides, such as oxyfluorfen and acifluorfen, but the Protox from *Bacillus subtilis* is known to be resistant to the herbicides (Jacobs et al. 1990, Dailey et al. 1994). Although *B. subtilis* Protox has similar kinetic characteristics to the eukaryotic enzyme which possesses a flavin and employs molecular oxygen as an electron acceptor, it is capable of oxidizing multiple substrates, such as Protox IX and coproporphyrinogen III. The mechanisms by which *B. subtilis* Protox exhibits resistance to the herbicides are still obscure, however its use for conferring herbicide resistant trait into crops would be particularly intriguing. The first trial for conferring the resistance against DPE herbicides by using the *B. subtilis* Protox gene was carried out in tobacco plants resulting in a 2-fold enhanced resistance at the 100 μM oxyfluorfen when judged by the cellular leakage and Chl loss (Choi et al. 1998). In spite of some degree of resistance in the transgenic tobacco plants, the constitutive expression of *B. subtilis* Protox gene in the cytoplasm arose question about the mechanism by which *B. subtilis* Protox awarded the herbicide resistance, since endogenous tobacco Protox which is supposed to be affected by the herbicide is localized at the plastid. Therefore, the question of whether expression of *B. subtilis* Protox gene in either the cytoplasm...
or the plastid confers the herbicide resistance into another important crop is needed to be answered.

This paper describes the generation of transgenic rice plants expressing a \textit{B. subtilis} \textit{Protox} gene via \textit{Agrobacterium}-mediated transformation. The transgenic rice plants were resistant to DPE herbicide oxyfluorfen at \textit{T}_0 generation. Furthermore, the transgenic rice lines of plastid targeted appeared to be more resistant to the herbicide than those of cytoplasm targeted.

**Materials and Methods**

\textbf{Transformation vector construction—} Two types of \textit{B. subtilis} \textit{Protox} gene constructs (Fig. 1) were prepared using conventional molecular biology techniques. A pGA1611:C vector was constructed to express the \textit{B. subtilis} \textit{Protox} gene in the cytoplasm. The full length of PCR amplified \textit{B. subtilis} \textit{Protox} gene was digested with \textit{SacI} and \textit{KpnI} and ligated into pGA1611 binary vector predigested with the same restriction enzymes resulting in placing the \textit{Protox} gene under the control of the maize ubiquitin promoter. The other construct (pGA1611:P) was designed to target the \textit{B. subtilis} \textit{Protox} into the plastid. For this purpose, PCR strategy was employed using specific primers which were designed according to the sequence data of tobacco (\textit{Nicotiana tabacum} \textit{cv. Samsun NN}; GenBank accession number Y13446) \textit{Protox}. The \textit{transit} peptide was amplified using the forward primer harboring a \textit{HindIII} site (underlined) 5-dTATCAAGCTTATGACAACAAACTCCCCAT)-3', a reverse primer 5-dATTGGAGCTCGGAGCATCGTGTTCTCCA)-3' harboring a \textit{SacI} site (underlined), and tobacco (\textit{N. tabacum} \textit{cv. KY160}) genomic DNA as a template. The PCR product was digested with \textit{HindIII} and \textit{SacI}, gel purified, and ligated into the same restriction sites within the pBluescript (Strategene). After verifying the sequence integrity, the \textit{HindIII} and \textit{SacI} fragment of transit sequence was ligated into the same restriction enzyme sites of pGA1611:C vector leading to the construction of pGA1611:P which has placed the transit peptide in front of the \textit{B. subtilis} \textit{Protox} gene.

\textbf{Transformation and regeneration—} Both \textit{A. tumefaciens} LBA4404 harboring pGA1611:C and pGA1611:P were grown overnight at 28°C in YEP medium supplemented with 5 mg ml\(^{-1}\) tetracyclin and 40 mg ml\(^{-1}\) hygromycin. The cultures were spun down and pellets were resuspended in an equal volume of AA medium containing 100 mg ml\(^{-1}\) acetosyringone. The calli were induced from scutellum of rice (\textit{Oryza sativa} \textit{cv. Naccdong}) seeds on N6 media as previously described (Rashid et al. 1996, Hei et al. 1997). The compact calli of 3–4 weeks old were soaked in a growth chamber at 25°C in darkness for 12 h and then exposed to continuous white light at 250 \textmu mol m\(^{-2}\) s\(^{-1}\) photosynthetically active radiation (PAR) for various time periods. No detrimental effects of acetone alone on the tissues were detected during the experiments (data not shown). Cellular leakage was determined periodically by detection of electrolyte leakage into the bathing medium using a conductivity meter (Cole-Parmer Instruments Co.) as previously described (Lee et al. 1995). Because of differences in background conductivity of different treatment solutions, results were expressed as changes in conductivity upon exposure to light. Previous studies have shown that oxyfluorfen has no effect on cellular leakage in darkness (Lydon and Duke 1988). All treatments for each measurement were triplicated.

\textbf{Chi determination—} The tissues were treated with oxyfluorfen and incubated in the same manner as used for the measurements of cellular leakage. Chi content was determined after 24 h of exposure to 250 \textmu mol m\(^{-2}\) s\(^{-1}\) PAR at 25°C. Chi 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 ml of dimethyl sulfoxide at room temperature. Chi extraction was complete at this time. Total Chi content in extracts was determined spectrophotometrically.

\textbf{Lipid peroxidation—} Lipid peroxidation was estimated by the level of malondialdehyde (MDA) production using a slight modification of the thiobarbituric acid (TBA) method as previously described (Buege and Aust 1978, Slater 1984). The tissues were treated with oxyfluorfen and incubated in the same manner as used for the measurements of cellular leakage and Chi. However, sucrose was omitted from the bathing medium because it is known to interfere with color development in the assay (Buege and Aust 1978, Kenyon et al. 1985). After 24 h of exposure to 250 \textmu mol m\(^{-2}\) s\(^{-1}\) PAR at 25°C, the treated tissues were separated from the bathing medium and both fractions were kept in a freezer at −80°C to avoid further reaction of the tissues with the herbicide until the MDA determination.

The tissues were homogenized with a mortar and pestle in 5 ml of a solution of 0.5% TBA in 20% trichloroacetic acid (TCA). The homogenate was centrifuged at 20,000 \times g for 15 min and the supernatants were collected. The supernatants were heated in a boiling water bath for 25 min and allowed to cool in an ice bath. Following centrifugation at 20,000 \times g for 15 min, the resulting supernatants were used for spectrophotometric determination of MDA. The aliquots of the bathing medium in which the tissues were incubated with different concentrations of oxyfluorfen were also subjected to the same procedure used for the tissues, using a
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\[ \text{pGA1611:C} \]

\[ \begin{array}{c}
\text{Ubi promoter} \quad \text{B. subtilis Protox gene} \quad \text{NOS} \quad \text{CaMV 35S-HPT-NOS}
\end{array} \]

\[ \text{pGA1611:P} \]

\[ \begin{array}{c}
\text{Ubi promoter} \quad \text{Ts} \quad \text{B. subtilis Protox gene} \quad \text{NOS} \quad \text{CaMV 35S-HPT-NOS}
\end{array} \]

Fig. 1 Schematic diagram of T-DNA region in binary vector. pGA1611:C and pGA1611:P were used for the transformation of rice calli. Ubi, ubiquitin; Ts, transit sequence; NOS, nopaline synthase terminator; CaMV 35S, cauliflower mosaic virus 35S promoter; HPT, hygromycin phosphotransferase.

1/1 (v/v) ratio of the aliquot to the solution of 0.5% TBA in 20% TCA. Absorbance at 532 nm for each sample was recorded and corrected for nonspecific turbidity at 600 nm. MDA concentrations were calculated using a molar extinction coefficient of 156 mM⁻¹ cm⁻¹ (Buege and Aust 1978). The MDA concentrations on a fresh weight basis from both fractions of the tissues and the bathing medium were pooled and then regarded as a total MDA produced by the tissues.

Results and Discussion

**PCR cloning of the transit sequence from tobacco Protox**—The sequence information of PCR-fished transit sequence showed a 189 nucleotides in length with 63 amino acids which has 11 amino acids longer than those of the reported tobacco Protox (Lermontova et al. 1997). Both deduced amino acid sequences were almost identical except the 12 consecutive stretch of serine residues in PCR-fished transit peptide (Fig. 2). However, the sequence variation seemed to be ascribed to the different cultivar of tobacco plants used as a template. The sequence had the common properties of transit peptide such as the richness of Ser/Thr and deficiency of Asp/Glu/Tyr (von Heijne et al. 1989).

**Transformation of rice plants**—Two gene constructs of pGA1611:C and pGA1611:P (Fig. 1) were employed to transform rice plants. These gene constructs were subcloned into a binary vector pGA1611 harboring a constitutive ubiquitin promoter which is known to be highly expressed in rice plants and have a hygromycin phosphotransferase as a selection marker and transformed into A. tumefaciens LBA4404. The scutellum-derived calli from rice seeds were co-cultivated with the A. tumefaciens harboring the above constructs. On average, 10–15% calli were survived from the selection medium containing 50 µg ml⁻¹ hygromycin. After transferring onto a regeneration

(A) ATGACAACAACCTCCACCCATGCAATCATTCTAATATATTTTC
ACCTCACCGGTACCGCGGTCCCTCCTCCCTCTCTCCCTCC
TCCTCCTCCTCCTCCCTCCATCGGCATTCTTAACTCGTACG
AGTTTCCTCCCTTCTCTTTCCATCTCGAAGCGCAATAGT
GTCAATTCGCAATGGCTGGAAACACCGATGCTCCGAGCTC

(B)

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<th>PCR (KY160)</th>
<th>Protox (Samsun)</th>
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<td>(^1\text{MTTTPIANHPNIFTHRSPPSSSSSSSSSSSSSSP} )</td>
<td>(^1\text{MTTTPIANHPNIFTHQQSSSSP} )</td>
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<tr>
<td>( \text{PCR (KY160)} )</td>
<td>( \text{Protox (Samsun)} )</td>
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<tr>
<td>( \text{SAFLTRSTAPFSSSKRNSVNSNGWRTRCS}^{53} )</td>
<td>( \text{LAFLNRTSFIPFSSSKRNSVNCNGWRTRCS}^{52} )</td>
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Fig. 2 Comparison of nucleotide sequence (A) and deduced amino acid sequence (B) of Protox transit peptides. Sacl primer site designed for the convenient subcloning was underlined. Sequence of tobacco \((N.\ tabacum \ cv. \ Samsun)\) Protox was derived from GenBank database (accession number Y13465). The PCR-cloned transit sequence has been deposited in GenBank (accession number AF225963).
medium, selected calli were regenerated into shoots at a rate of 1–5%. During the process of regeneration, some young shoots emerged from the plastid targeted lines (pGA1611:P) were inclined to be etiolated under normal light intensity. However, this phenomenon could be overcome by growing them under dim light condition (ca. 20 μmol m$^{-2}$ s$^{-1}$ PAR) for 1 week and subsequently transferring them under normal light condition, in which the shoots began to grow normally without being etiolated. It could be explained that these transgenic lines due to the possible overexpression of the *B. subtilis* Protox gene in the plastid are oxidizing Protogen IX into Proto IX, an enzymatic product beyond the optimal level of Proto IX, which are required for the downstream metabolic process, leading to phototoxicity to plant cells (data not shown). On the whole, 6 and 58 different transgenic rice lines having pGA1611:C and pGA1611:P constructs expecting to be expressed the *B. subtilis* Protox gene in the cytoplasm or in the plastid, respectively, were grown to maturity. Most of the transgenic lines appeared to have normal phenotypes, but their seed production varied ranging from 4 to 260 seeds depending on the individual transgenic lines.

**Genomic DNA gel blot analysis**—To assess the stable integration of the *B. subtilis* Protox gene into the rice genome of the transgenic lines regenerated from the hygromycin selection medium, DNA was extracted separately from 5 transgenic lines of cytoplasm targeted and 6 transgenic lines of plastid targeted, digested with HindIII, and hybridized with 32P-labeled *B. subtilis* Protox gene. Due to the absence of HindIII site within the probed transgene, the number of hybridized bands directly corresponded to the copy number of transgene in genome of the transgenic lines. The cytoplasm targeted transgenic lines (C2, C5, and C6) showed the multiple bands around three hybridizing bands each above 5 kb in size, suggestive of multiple insertions of the transgene at different locations in the rice genome (Fig. 3). In contrast, lines C1 and C3 have single copy insertion in the rice genome. As for the plastid targeted lines, 5 out of 6 plastid targeted lines had single copy insertion except the line P15 showing three copy insertion.

![Genomic DNA blot analysis](image1)

**Fig. 3** Genomic DNA blot analysis of the *B. subtilis* Protox transgene in transgenic rice lines at *T$_o$* generation. W, nontransgenic rice plants; C1–C6, transgenic lines of cytoplasm targeted; P1–P15, transgenic lines of plastid targeted.

![RNA gel blot analysis](image2)

**Fig. 4** RNA gel blot analysis of the *B. subtilis* Protox mRNA levels in transgenic rice lines at *T$_o$* generation. W, nontransgenic rice plants; C1–C6, transgenic lines of cytoplasm targeted; P1–P15, transgenic lines of plastid targeted.
RNA gel blot analysis—*B. subtilis* Protox mRNA was detected in total RNA isolated from the leaves of both the cytoplasm targeted and the plastid targeted transgenic lines, whereas no *B. subtilis* Protox mRNA was detected in total RNA prepared from the nontransgenic rice leaves (Fig. 4). In the cytoplasm targeted lines, C1 and C3 had very low levels of the *B. subtilis* Protox mRNA expression, while lines C2, C5, and C6 expressed relatively high levels of the *B. subtilis* Protox mRNA. As for plastid targeted lines, all the lines tested were able to transcribe the *B. subtilis* Protox gene, in which line P15 exhibited the highest level of the transgene expression. In the light of some relevance between the copy number of transgene and relative mRNA expression level, the level of the *B. subtilis* Protox mRNA expression appeared to be associated with the copy number of the transgene in the rice genome.

Evaluation of oxyfluorfen resistance of the transgenic rice plants—Physiological responses of the nontransgenic and the transgenic rice plants to oxyfluorfen were compared with respect to cellular leakage, Chl loss, and lipid peroxidation, which have usually been used for assessing the effects of peroxidizing herbicides (Kenyon et al. 1985, Becerril and Duke 1989, Lee et al. 1995). The representative transgenic rice plants targeted to the cytoplasm (lines C1, C2, C5, and C6) and targeted to the plastid (lines P1, P3, P4, and P15) were employed for this purpose.

Oxyfluorfen caused considerable cellular leakage from the treated leaf squares of the nontransgenic rice plants upon the exposure of light following 12 h incubation in darkness (Fig. 5). The cellular leakage increased depending on the concentration of the herbicide and the duration of incubation time. However, the magnitude of the cellular leakage was much lower from the transgenic than from the nontransgenic rice leaf squares treated with varying concentrations of oxyfluorfen (Fig. 5). The cellular leakage from leaf squares of the transgenic plants targeted to the cytoplasm greatly varied with the lines. Some of the transgenic plants targeted to the cytoplasm did not exhibit the resistance to oxyfluorfen. For example, the cellular leakage from leaf squares of the line C1 was almost com-

![Fig. 5](https://academic.oup.com/pcp/article-abstract/41/6/743/1923284)

**Fig. 5** Effect of oxyfluorfen on cellular leakage from leaf squares of the nontransgenic and the transgenic rice plants. W, nontransgenic rice plants; C1–C6, transgenic lines of cytoplasm targeted; P1–P15, transgenic lines of plastid targeted. The tissues were exposed to continuous light at 250 μmol m⁻² s⁻¹ at 25°C for 12 h following 12 h dark incubation.

![Fig. 6](https://academic.oup.com/pcp/article-abstract/41/6/743/1923284)

**Fig. 6** Effect of oxyfluorfen on Chl loss from leaf squares of the nontransgenic and the transgenic rice plants. W, nontransgenic rice plants; C1–C6, transgenic lines of cytoplasm targeted; P1–P15, transgenic lines of plastid targeted. The tissues were exposed to continuous light at 250 μmol m⁻² s⁻¹ at 25°C for 12 h following 12 h dark incubation.
parable to that of the nontransgenic plants upon the oxyfluorfen treatment. In response to the oxyfluorfen treatment, the transgenic rice plants targeted to the plastid exhibited much lesser cellular leakage than the transgenic plants targeted to the cytoplasm (Fig. 5).

Similar tendency was obtained with Chl loss and lipid peroxidation in the leaf tissues of the nontransgenic and the transgenic rice plants following the treatment of oxyfluorfen. Oxyfluorfen effectively reduced Chl content in leaves from the nontransgenic rice plants in a concentration-dependent manner, but little or no effects were observed in the treated leaves from the transgenic rice plants targeted to the cytoplasm or to the plastid (Fig. 6). Oxyfluorfen also caused significant lipid peroxidation in the treated leaves of the nontransgenic rice plants (Fig. 7). Following the oxyfluorfen treatment, much lesser degree of lipid peroxidation was detected in the transgenic rice plants targeted to the cytoplasm than in the nontransgenic rice plants and even no lipid peroxidation occurred in the transgenic rice plants targeted to the plastid (Fig. 7).

The results of the cellular leakage, Chl loss, and lipid peroxidation indicate that the transgenic rice plants expressing the B. subtilis Protox gene are resistant to oxyfluorfen. Furthermore, the transgenic rice plants targeted to the plastid exhibited higher resistance to the herbicide than the transgenic plants targeted to the cytoplasm.

Possible resistance mechanisms in the transgenic rice plants to DPE herbicides—The resistance mechanism in the transgenic plants is not known due to the complicated action mechanism of DPE herbicides. However, the resistance could be explained if, in the transgenic rice plants targeted to the cytoplasm, Protogen IX outside the plastid is oxidized into Proto IX in the cytoplasm rather than in the plasma membrane (PM), although PM has been thought to be the major site of Protogen IX oxidation to Proto IX and of subsequent Proto IX accumulation in normal plant tissues upon treatment with DPE herbicides (Jacobs and Jacobs 1993, Lee et al. 1993, Duke et al. 1994, Lee and Duke 1994). Because of high lipophilicity of Proto IX (Lee et al. 1993), it could be migrated from the cytoplasm into the plastid and the mitochondrion, and then be further metabolized to later intermediates of the Chl or heme biosynthetic pathway. In fact, the reentering of Proto IX from the cytoplasm into the normal Chl or heme biosynthetic pathway in the plastid and the mitochondrion has been proposed (Duke et al. 1991, Smith et al. 1993). Proto IX could also be partitioned into the PM, which is devoid of chelatase activities (Duke et al. 1991, 1994), but the Proto IX accumulation in the PM could much be alleviated. This fact might account for the resistance of the transgenic rice plants targeted to the cytoplasm.

In the transgenic rice plants targeted to the plastid, on the other hand, B. subtilis Protox expressed in the plastid will oxidize Protogen IX to Proto IX regardless of the presence of DPE herbicides, presumably at higher rate than the endogenous plastid Protox in the absence of the herbicides. Although the intermediates of the Chl or heme biosynthetic pathway beyond Protogen IX will be accumulated in the plastid membrane and act as photosensitizers, this phenomenon might be overcome by feedback inhibition of d-aminolevulinic acid synthesis, the committed step of the pathway, by heme and protochlorophyllide (Beale and Weinstein 1990, Becerril et al. 1992). Thus, the transgenic plants targeted in the plastid will be resistant to DPE herbicides and accomplish normal Chl or heme biosynthesis. However, the proposed resistance mechanisms in the transgenic plants targeted to the cytoplasm or to the plastid should further be substantiated in the near future.

Taken altogether, the transgenic rice plants were generated via expression of B. subtilis Protox gene under ubiquitin promoter targeted to the cytoplasm or to the plastid using Agrobacterium-mediated gene transformation. The integration and expression of the transgene were successfully accomplished in the transgenic plants and the plants exhibited the resistance to DPE herbicide oxyfluorfen at T0 generation.

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Fig. 7 Effect of oxyfluorfen on MDA production in leaf squares of the nontransgenic and the transgenic rice plants. W, nontransgenic rice plants; C6, transgenic line of cytoplasm targeted; P15, transgenic line of plastid targeted. The tissues were exposed to continuous light at 250 μmol m⁻² s⁻¹ at 25°C for 12 h following 12 h dark incubation.

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