Note

Generation of Resistance to the Diphenyl Ether Herbicide, Oxyfluorfen, via Expression of the *Bacillus subtilis* Protoporphyrinogen Oxidase Gene in Transgenic Tobacco Plants

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Received June 23, 1997

In an effort to develop transgenic plants resistant to diphenyl ether herbicides, we introduced the protoporphyrinogen oxidase (EC 1.3.3.4) gene of *Bacillus subtilis* into tobacco plants. The results from a Northern analysis and leaf disc assay indicate that the expression of the *B. subtilis* protoporphyrinogen oxidase gene under the cauliflower mosaic virus 35S promoter generated resistance to the diphenyl ether herbicide, oxyfluorfen, in transgenic tobacco plants.

**Key words:** diphenyl ether herbicide; protoporphyrinogen oxidase; *Bacillus subtilis*; oxyfluorfen; transgenic tobacco plant

Such diphenyl ether herbicides as oxyfluorfen exert their herbicidal effect by causing rapid photodegradation and desiccation of plant tissues.1-3 The primary target of the herbicides is known to be protoporphyrinogen oxidase (Protox), the last common enzyme in the biosynthesis of hemes and chlorophylls.4-7 The biochemical basis for the herbicidal mechanism is the competitive inhibition of plastid Protox, whereby protoporphyrinogen IX (Proton IX), the substrate of the enzyme, is accumulated in the plastid envelope, diffused into the cytosol, and transported to the plasma membrane (PM), where it is rapidly oxidized to protoporphyrin IX (Proto IX) by a herbicide-insensitive peroxidase-like enzyme8-10 and partly by its autooxidation.11 Resulting Proto IX causes photodynamic membrane lipid peroxidation and ultimate cellular death in the presence of molecular oxygen and light.12-13,14

The resistance of plant species to herbicides can theoretically be evolved in various ways, including reduced uptake or sequestration of the herbicides, and rapid metabolic destruction of the herbicides, Proton IX, and/or Proto IX. These types of resistance have been found in natural resistance of several plant species.12-14 The development of transgenic plants resistant to diphenyl ether herbicides appears to be possible, since Protox originated from some prokaryotes is poorly inhibited by the herbicides.15,16 Furthermore, the Protox gene from *B. subtilis* has recently been cloned and expressed in *E. coli*.10 In an effort to develop transgenic plants, we introduced the Protox gene of *B. subtilis* into tobacco plants. We demonstrate in this study that the expression of the Protox gene of *B. subtilis* generated resistance to the diphenyl ether herbicide, oxyfluorfen, in transgenic tobacco plants.

Oxyfluorfen (95% pure) was a generous gift from Rohm and Haas Co. Restriction enzymes and DNA modifying enzymes were from Promega and Sigma Chemical Co. All other reagents were of the highest quality commercially available. The Protox gene was isolated from the genomic library of *B. subtilis* (Clontech) by standard procedures7 and amplified by PCR with primers of 5′-GGCGAAGCTTGGATCCATGAGTGACCAGAAA-3′ (N-terminal) and 5′-GGCGAGCTTTAGCTGAAATAAT-3′ (C-terminal). The 1.4 kb PCR product was digested with BamHI and inserted in the sense orientation between the cauliflower mosaic virus (CaMV) 35S promoter and the terminator of the nopaline synthase (NOS) gene of the pBI121 vector (Fig. 1A). The promoter and signal peptide region of the chlorophyll a/b binding protein (Cab) gene was cloned from potato leaves by PCR with primers of 5′-GGCGAAGCTTGGATCCATGAGTGACCAGAAA-3′ (N-terminal) and 5′-GGCGGATCCCTTCATGGAATG3′ (C-terminal).18 The PCR product was ligated with the Protox gene of *B. subtilis*, and the resulting Cab promoter/signal sequence-Protox gene (1.6 kb) substituted for the CaMV 35S-promoter-NOS region of the pBI121 vector (Fig. 1B). These constructs were used to transform tobacco leaf segments via Agrobacterium. The resulting transgenic plants were screened by PCR and a Southern analysis with the Protox gene (1.4 kb) as a probe. We expected that the Protox gene under the CaMV 35S promoter would be expressed in the cytosol and Cab promoter/signal se-

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**Abbreviations:** Protox, protoporphyrinogen oxidase; Proton IX, protoporphyrinogen IX; PM, plasma membrane; Proto IX, protoporphyrin IX; CaMV, cauliflower mosaic virus; NOS, nopaline synthase; Cab, chlorophyll a/b binding protein.
The PCR product of the Protopx gene from *B. subtilis* was inserted between (A) the CaMV 35S promoter or (B) the potato Cab promoter/signal sequence and the NOS gene.

sequence would direct the expression of the Protopx gene to the chloroplast.

The transgenic plants containing the *B. subtilis* Protopx gene (#3 and #16, under the CaMV 35S promoter; #C1, #C2 and #C3, under the Cab promoter/signal sequence) were further subjected to a Northern analysis. The Northern analysis, using the Cab-promoter/signal sequence-Protopx gene (1.6 kb) as a probe, showed that the Protopx gene of *B. subtilis* was expressed in the transgenic tobacco plants under both the CaMV 35S and Cab promoters (Fig. 2).

The oxyfluorfen resistance of the transgenic tobacco plants was evaluated by using the leaf disc assay as reported. Tobacco tissues from the third and fourth true leaves were treated with various concentrations of oxyfluorfen by cutting fifty 4-mm diameter leaf discs with a cork borer and then placing them into a polystyrene Petri dish (6-cm diameter) in 5 ml of 1% sucrose and 1 mM 2-(N-morpholino)ethanesulfonic acid (pH 6.5) with or without oxyfluorfen dissolved in acetone. The discs were then incubated at 25°C in darkness for 12 h before being exposed to 250 µmol/m²/sec of photosynthetically active radiation for 8 h. Cellular damage was determined by detecting electrolyte leakage into the bathing medium with a conductivity meter (Cole-Parmer Instruments Co.). Because of differences in the background conductivity of different treatment solutions, the results are expressed as the changes in conductivity from initial measurements. The experiments were repeated, and the results shown are the means of triplicate treatments from representative experiments. Compared to the nontransgenic tobacco plants, the transgenic tobacco plants under the CaMV 35S promoter (#3 and #16) appeared to be more resistant to oxyfluorfen in terms of photobleaching and electrolyte leakage caused by oxyfluorfen. The results are shown in Fig. 3.
by oxyfluorfen (Figs. 3 and 4). The transgenic tobacco plants under the Cab promoter/signal sequence (#C1, #C2 and #C3), however, exhibited a greatly reduced growth rate and chlorophyll level (data not shown). Based on an electron microscopic analysis, malformation of thylakoid membranes was also obvious in the transgenic tobacco plants of #C1, #C2 and #C3 (data not shown).

In conclusion, the expression of the Protox gene of B. subtilis under the CaMV 35S promoter generated resistance to oxyfluorfen in the transgenic tobacco plants. The resistance mechanism in the transgenic tobacco plants is not known due to the complicated action mechanism of diphenyl ether herbicides. However, the resistance could be explained if, in the transgenic tobacco plants, Protagen IX outside the plastids is oxidized into Proto IX in the cytosol rather than in PM, although PM has been thought to be the major site of Protagen IX oxidation to Proto IX and of subsequent Proto IX accumulation in normal plant tissues upon treatment with diphenyl ether herbicides.3,7,8,9) Proto IX in the cytosol might be metabolized to later intermediates of the chlorophyll or heme biosynthetic pathway after reentering plastids and mitochondria, and thus the actual concentration of photodynamic Proto IX would be reduced within the cell. In contrast, the chance of Proto IX in PM being further metabolized would be much lower, since Proto IX is known to be a hydrophobic molecule9) and PM is devoid of chelatase activities.3,7,8) Only after a sufficient level of Proto IX has accumulated in PM, could Proto IX reenter the chlorophyll or heme biosynthetic pathway.7) In fact, reentering of Proto IX from the cytosol into the normal chlorophyll or heme biosynthetic pathway in plastids7) and mitochondria9) has been proposed.

Acknowledgments

This work was assisted by support from Korean Ministry of Education to O. Han. Rohm and Haas Co. generously provided technical-grade oxyfluorfen.

References