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BRIEF COMMUNICATION

Transgenic rice plants expressing *Bacillus subtilis* protoporphyrinogen oxidase gene show low herbicide oxyfluorfen resistance

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Abstract

Transgenic rice plants harbouring *Bacillus subtilis* protoporphyrinogen oxidase (Protox) gene, which is targeted into plastid, were generated by *Agrobacterium*-mediated transformation using a rice (*Oryza sativa* L. cv. Dongjin) and their gene integration at T_1 generation by Southern and mRNA expression in T_2 generation by Northern blotting were analyzed. Their herbicide-resistant trait was further confirmed by *in vitro* leaf segment assay and *in planta* bioassays such as seed germination assay and measurement of growth inhibition. The herbicide oxyfluorfen resistance in transgenic rice plants was not very high. The results showed that the Protox from *B. subtilis* can not be applicable as a gene source to generate a high level oxyfluorfen tolerance in plants.

Additional key words: diphenylether herbicide, herbicide-resistant transgenic rice, leaf disc assay, *Oryza sativa.*

The primary target site of diphenylether (DPE) herbicides, such as oxyfluorfen, acifluorfen, is protoporphyrinogen oxidase (Protox), an enzyme of chlorophyll and heme biosynthetic pathway (Matringe *et al.* 1989, Beale and Weinstein 1990). The Protox catalyzes the oxidation of protoporphyrinogen IX (Protogen IX) to protoporphyrin IX (Proto IX). It has been known that inhibition of Protox leads to the accumulation of a colourless substrate of the enzyme, Protoporphyrinogen IX, which apparently diffuses from the chloroplast into the cytosolic compartment where it is oxidized nonspecifically by herbicide resistant plasma membrane peroxidases (Matringe *et al.* 1989). Therefore, Protox inhibition leads to an accumulation of Proto IX, the first light-absorbing chlorophyll precursor. Light absorption by Proto IX apparently produces triplet state Proto IX that interacts with ground state oxygen to form singlet oxygen. Both triplet Proto IX and singlet oxygen can abstract hydrogen from unsaturated lipids resulting in a chain reaction of lipid peroxidation (Girotti 1990). Due to a well known resistance of *B. subtilis* Protox against DPE herbicide (Dailey *et al.* 1994, Corrigall *et al.* 1998), the *Protox* gene cloned from *B. subtillis* had been used for developing herbicide resistant transgenic plants. The first trial was transgenic tobacco plants that had shown to be resistant to herbicide oxyfluorfen when evaluated only *in vitro* by either cellular leakage analysis or leaf disk assay (Choi *et al.* 1998). Transgenic rice plants expressing *B. subtilis Protox* genes also proved to be resistant *in vitro* assay to herbicide oxyfluorfen (Lee *et al.* 2000). However, precise measurement of *in planta* resistance of their transgenic plants against herbicide have been hindered possibly due to either higher herbicidal activity of DPE type herbicides or lower herbicidal resistance of transgenic plants. Hence, this study was performed to determine whether transgenic plants of different rice cultivar (cv. Dongjin) expressing *B. subtilis Protox* gene in plastid exhibit herbicide resistant traits *in planta* or not.

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Abbreviations: Protox - protoporphyrinogen oxidase; DPE - diphenylether; Protogen IX - protoporphyrinogen IX; Proto IX - protoporphyrin IX.

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Agrobacterium tumefaciens LBA4404 harbouring a binary vector pGA1611:P which was designed to express a *B. subtilis* Protox targeted into plastid was used to deliver the *B. subtilis* Protox gene into rice genome. The scutella derived calli from rice cv. Dongjin (Visarada *et al.* 2002) were used for co-culture with *Agrobacterium*. Transgenic selection and regeneration were the same as described previously (Lee *et al.* 2000). As for DNA and RNA blot analysis, 5 μg of genomic DNA and 10 μg total RNA isolated from wild and transgenic rice leaves were used.

Using pGA1611:P construct which was designed to express the *B. subtilis* Protox targeted into plastids, we generated tens of independent transgenic rice plants using scutellum-derived calli of *Oryza sativa* cv. Dongjin with the standard transformation techniques (Hiei *et al.* 1997).

The transgenic rice plants harbouring the *B. subtilis Protox* gene were used for genomic DNA blot analysis in T_1 generation (Fig. 1*A*). All the lines tested had 1 to 3 copies of the *Protox* gene in rice genome. *B. subtilis Protox* mRNA in T_2 generation was expressed in all the lines analyzed. The expression seemed unproportional to the number of gene copies inserted into the genome (Fig. 1*B*). Two independent homozygous lines, P2 and P6, each of which had shown a single copy transgene insertion, were further selected for evaluating herbicide-resistance trait.

First, the herbicide-resistance of transgenic rice plants was analyzed by *in vitro* leaf segment assay. Rice tissues from the third and fourth true leaves were treated with various concentrations of oxyfluorfen by cutting 5-mm squares with a razor blade, and then placing them in a Petri dish containing 1 % sucrose and 1 mM MES (pH 6.5). The control contained the same amount of solvent without herbicide. The tissues were incubated in a growth chamber at sequence of 26 °C in darkness for 12 h and then exposed to continuous white light (250 μmol m^{-2} s^{-1} PAR at 28 °C). A photograph was taken 7 d after treatments.

The leaf segments of non-transgenic plant (W) were photobleached rapidly and exhibited a brownish colour. They greatly reduced chlorophyll content even in 1 μM oxyfluorfen, and above 100 μM, these leaf segments were totally bleached. In contrast to the non-transgenic plant, transgenic plants P2 and P6 were resistant to the various concentrations of oxyfluorfen and remained green for a long time without chlorosis (Fig. 2). Above 100 μM oxyfluorfen, the leaf fragments of the transgenic plants lost their resistance. This result is analogous to previous

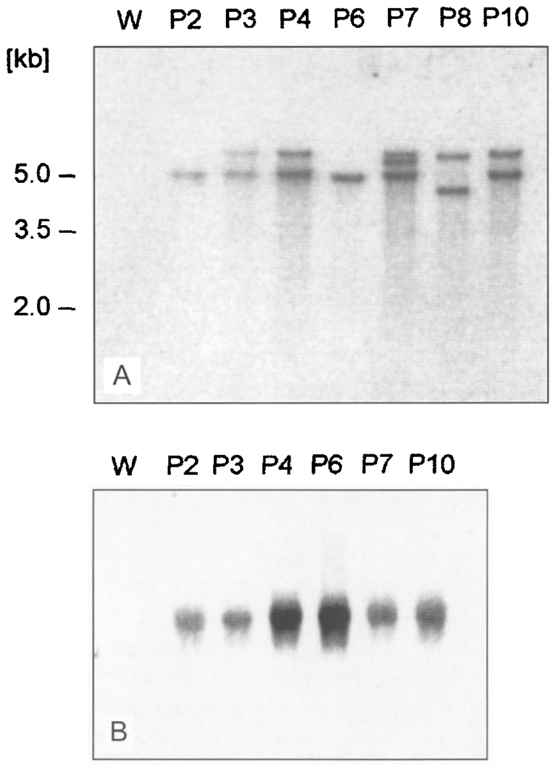

Fig. 1. Southern (*A*, T_1 generation) and Northern Blot (*B*, T_2 generation) analysis of transgenic lines. W - non-transgenic rice plant, P2 to P10 - transgenic rice plants.

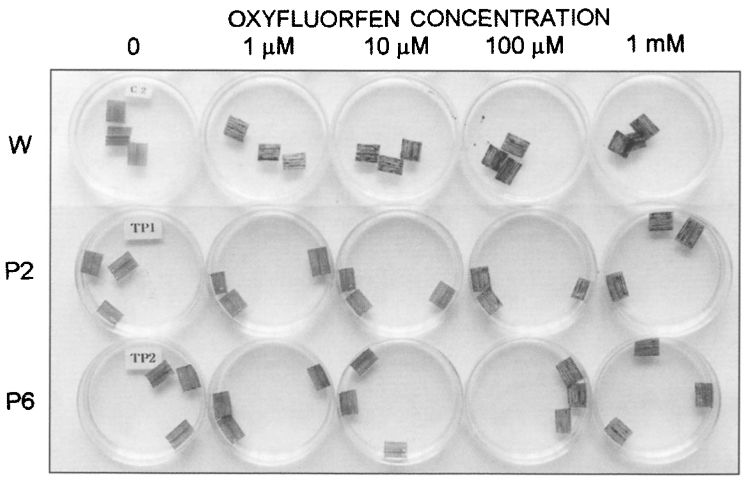

Fig. 2. Leaf segment assay in various concentration of oxyfluorfen. W - nontransgenic rice plant; P2 and P6 - transgenic rice plants.

data of chlorophyll loss upon oxyfluorfen treatment of the transgenic rice cv. Nakdong (Lee *et al.* 2000).

In order to do *in vivo* germination assay, the husked and sterilized rice seeds were planted on a half-strength MS medium containing 250 mg dm^{-3} cefotaxime. On top of the medium, 1 μM oxyfluorfen solution was added up to bring 2 cm in depth. These were incubated in 26 °C darkness for 2 d until germination. When seedlings reached the layer of oxyfluorfen, the bottle was exposed to continuous light (250 μmol m^{-2} s^{-1} PAR) at 28 °C and was further incubated for 1 d. In order to minimize the herbicidal effects on rice seedlings, all the oxyfluorfen solution dissolving in 1 % acetone (v/v) was drained out and filled with the same volume of sterilized water containing 1% acetone which does not inhibit rice germination and growth. From 5 d after draining out the oxyfluorfen solution, their herbicide-resistance was evaluated by measuring the shoot length and fresh mass (Fig. 3). Transgenic lines had grown vigorously while the growth of non-transgenic rice plants had been delayed. The average length and fresh masses of shoots and roots of transgenic rice plants was approximately twice as high as those of nontransgenic rice plants. The correlation of *Protox* mRNA content and *in vivo* herbicide tolerance was not observed between P2 and P6.

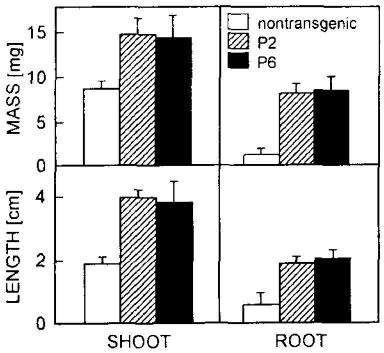

Fig. 3. Growth inhibition measurement by *in vivo* germination assay. The 30 seeds of the two lines were measured 10 d after incubation in vessels. Mean ± SD.

Thus far, two transgenic plants expressing *B. subtilis* Protox were generated from tobacco and rice (Choi *et al.* 1998, Lee *et al.* 2000). These transgenic plants showed only *in vitro* herbicide resistance when analyzed with their mature leaves. When oxyfluorfen was applied to rice plants at a matured or young leaf stage, there were no apparent herbicide resistance differences between transgenic and non-transgenic rice plants. Finally, we were successful in evaluating different herbicide resistance of transgenic and non-transgenic rice plants by using a seed germination assay. Surface-sterilized rice seeds were placed on a half-strength Murashige-Skoog solid medium containing 250 mg dm^{-3} cefotaxime in a glass jar (*Sigma*, St. Louis, USA). 0.025 cm^3 of 1 μM oxyfluorfen was added onto the top layer of the solid medium. The depth of the oxyfluorfen layer to the surface of the medium was set to about 2.0 cm, because the average extension of a rice shoot in 5 d generally was about 2.0 cm; moreover, this period coincided with the half-life of the herbicide in water, which is assumed to be as short as 5 d.

Results demonstrate that the transgenic rice plants transformed with the *B. subtilis Protox* gene were resistant to herbicide oxyfluorfen both *in vitro* and *in vivo*. However, these transgenic rice did not seem to exhibit a high enough herbicide resistance to survive in the presence of more than 2 μM oxyfluorfen which completely inhibits germination of non-transgenic rice seeds. Recently, it was reported that transgenic tobacco expressing *Arabidopsis Protox* gene showed resistance to DPE herbicide acifluorfen in which the transgenic tobacco seeds were able to germinate, while non-transgenic seeds did not in the presence of 300 nM acifluorfen (Lermontova and Grimm 2000). In a more practical point of view, the *B. subtilis* Protox, which was known as a resistant enzyme against DPE herbicides, is not a good target gene to obtain transgenic crops resistant to DPE herbicides due to its inability to exhibit a high level of herbicide resistance *in planta*.

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