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Use of *Myxococcus xanthus* protoporphyrinogen oxidase as a selectable marker for transformation of rice

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Abstract

Protoporphyrinogen oxidase (PPO) is the target enzyme of peroxidizing herbicides. The overexpression of *Myxococcus xanthus* PPO (*Mx* PPO) confers a high level of herbicide resistance in rice. Among the peroxidizing herbicides, butafenacil has an efficiency ~1000-fold that of oxadiazon, as judged by calli susceptibility tests upon herbicide treatment. Butafenacil (0.1 μM) was used to select transgenic rice plants expressing *Mx* PPO under the control of the constitutive maize ubiquitin promoter. The ectopic expression of the *Mx* PPO transgene was investigated in the T_0 generation by Northern blot and Western blot analysis. The T_0 transgenic plants expressing the *Mx* PPO gene were resistant to butafenacil based on in vitro leaf disk and in vivo foliar spray tests.

Keywords: Protoporphyrinogen oxidase; Butafenacil; Peroxidizing herbicides; Transgenic rice; Selectable marker gene

1. Introduction

In rice transformation, two selectable marker genes, the hygromycin B phosphotransferase and bialaphos-resistance gene (Bar), have been heavily used in combination with hygromycin and phosphinothricin as selective agents. Although a number of selectable marker systems are available for rice, new and convenient systems are in demand for transforming multiple genes and improving transformation efficiency [1]. Peroxidizing herbicides represent a broad class of herbicides comprising at least eight structural families, including more than 27 herbicides produced by several agrochemical companies [2]. These herbicides target the enzyme protoporphyrinogen IX oxidase (PPO)[1] and have been used commercially since 1960 to control annual grasses and dicotyledonous weeds in soybean (*Glycine max*), peanut (*Arachis hypogaea*), cotton (*Gossypium hirsutum*), rice (*Oryza sativa*), and other crops [3–5].

Diphenyl ethers, triazolinones, *N*-phenylpyrazoles, oxadiazoles, thiadiazoles, pyrimidindiones, *N*-phenylphthalimides, and oxazolidinediones are major structural families of herbicidal PPO inhibitors. As partial structural analogs of protoporphyrinogen IX, these herbicides bind to and competitively inhibit PPO activity [6]. New potent PPO inhibitors continue to be developed and are reported to be effective peroxidizing herbicides [7]. The inhibition of PPO results in the massive production of singlet oxygen, which is followed by peroxidation of membrane lipids and subsequent cell death [8,9].

Since the first report of a transgenic tobacco plant expressing an *Arabidopsis* PPO [10], many attempts have been made to develop transgenic crops resistant to peroxidizing herbicides through the ectopic expression of native or mutant forms of PPO [11–15]. An *Arabidopsis* double mutant of PPO and a *Myxococcus xanthus* (*Mx*) PPO were shown to confer a high level of resistance to peroxidizing herbicides in transgenic maize and rice, respectively [14,15]. Furthermore, the *Arabidopsis* double

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[1] *Abbreviations used*: PPO, protoporphyrinogen oxidase; *Mx*, *Myxococcus xanthus*.

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mutant of PPO was successfully used as a selectable marker gene, in combination with the herbicide butafenacil as a selection agent, for generating transgenic crops [14,16].

In this report, we describe the development of transgenic rice plants by using the *Mx* PPO gene as a selectable marker gene coupled with the herbicide butafenacil as a selection agent.

2. Materials and methods

2.1. Calli viability test

Scutellum-derived healthy and compact calli from wild-type plants (*O. sativa* cv. Dongjin) were placed on N6 medium containing 2,4-dichlorophenoxyacetic acid (2,4-D) [12] and various concentrations of peroxidizing herbicides for 7 days at 28 °C in the dark. Afterward, the calli were incubated at 28 °C under a cycle of 12-h light/12-h dark for 3 days. Tetrazolium was used to determine calli viability. Tetrazolium Red (Sigma–Aldrich, St. Louis, MO) was dissolved in 0.05 M potassium phosphate buffer (pH 4.9), and the calli were soaked for 3 days at 28 °C in the dark. The calli were briefly washed in distilled water and photographed. Live calli turned red, whereas dead calli remained white or light yellow. Technical-grade oxyfluorfen and oxadiazon were generously provided from Kyungnong Co. (Kyungju, Korea) and Hankooksamgong Co. (Seoul, Korea), respectively. Acifluorfen was obtained from Fluka (Seoul, Korea). Technical-grade carfentrazone and butafenacil were gifts from Dr. Guh (Chonnam National University, Korea).

2.2. Vector construction and rice transformation

Agrobacterium-mediated transformation was used to generate transgenic rice plants. The full-length cDNA of *Mx* PPO [13; a gift from Dr. H.A. Dailey, University of Georgia, Athens, GA] was amplified by PCR using the cDNA clone as a template and the following primer set: forward, 5′-TATCAAGCTT**ATG**CACCACATGCCGAGGA-3′ (underlined, *Hin*dIII restriction site; bold, translation start codon), and reverse, 5′-TCAGGGTCACCCTACGGGGCGTGGGAG GT-3′ (underlined, *Bst*EII site). The PCR product was digested with *Hin*dIII and *Bst*EII and was gel-purified. The maize ubiquitin promoter was excised from pGA1611 binary vector [17] using *Bam*HI and *Hin*dIII and was gel-purified. To generate the pCAMBIA0380:*Mx* PPO construct, the *Bam*HI–*Hin*dIII fragment of the ubiquitin promoter and the *Hin*dIII–*Bst*EII fragment of *Mx* PPO were ligated into the pCAMBIA0380 binary vector (CAMBIA Center, Canberra, Australia) digested with *Bam*HI and *Bst*EII (Fig. 2). This vector carries the *Mx* PPO gene as a transgenic selectable marker. The resulting pCAMBIA:*Mx* PPO binary vector was transformed into *Agrobacterium tumefaciens* LBA4404, which in turn was co-cultured with scutellum-derived rice calli (*O. sativa* cv. Dongjin). For the selection process, the calli were grown in the presence of 0.1 μM butafenacil for 1 week in darkness and then placed under light (12-h light/12-h dark) for 3 days to allow the herbicidal action of butafenacil, followed by further selection for 3 weeks in darkness at 28 °C. Subsequent regeneration procedures were the same as described previously [12].

2.3. Isolation and analysis of nucleic acids

Total RNA was extracted using TRI Reagent (Sigma), and 10 μg total RNA were fractionated in a 1.0% agarose–formaldehyde gel with 20 mM MOPS as the electrophoresis buffer. The RNA bands were blotted onto a nylon membrane and hybridized with a *Mx* PPO cDNA probe. Equal loading of the RNA samples was confirmed by ethidium bromide staining of the gel prior to blotting. Hybridization was performed as previously described [12].

2.4. Western blot analysis

For the immunoblot analysis, rice leaves (0.2 g) were homogenized, using a mortar and pestle, with 1 ml of extraction buffer (80 mM Tris–HCl [pH 7.0], 15 mM $MgCl_2$, 10 mM sodium metabisulfite, 10 mM sodium ascorbate, 20% glycerol, 1% PVP) and 0.1 of a Mini Complete Protease Inhibitor tablet (Boehringer–Mannheim, Mannheim, Germany). The homogenates were centrifuged for 10 min at 10,000 g at 4 °C, and the supernatant fractions were analyzed for protein. The protein extracts (50 μg) were fractionated by electrophoresis in an 11.5% (w/v) acrylamide/bis gel containing SDS, transferred to a PVDF membrane, and immunoblotted with *Mx* PPO polyclonal antibodies [15]. To detect immunoreactivity, an ECL Western blotting kit (Boehringer Mannheim) was used according to the manufacturer's instructions.

2.5. Leaf disk and foliar spray assays for butafenacil resistance

Leaves of T_0 transgenic and wild-type plants were treated with various concentrations of butafenacil. Five leaf disks, 4 mm in diameter, were cut using a cork borer and placed into a polystyrene Petri dish (diameter, 2.5 cm) containing 1 ml of 1% sucrose and 1 mM 2-(*N*-morpholino) ethanesulfonic acid (pH 6.5) with or without butafenacil. The disks were incubated at 28 °C in darkness for 12 h before being exposed to 250 $\mu mol\, m^{-2}\, s^{-1}$ photosynthetically active radiation for 5 days. Transgenic T_0 and wild-type seedlings were used for the foliar application test. The rice plants were sprayed with 5 μM technical-grade butafenacil, placed in a growth chamber at 28 °C in darkness for 12 h, and then exposed to continuous white light (250 $\mu mol\, m^{-2}\, s^{-1}$ photosynthetically active radiation) for 5 days.