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Modifying *Myxococcus xanthus* protoporphyrinogen oxidase to plant codon usage and high level of oxyfluorfen resistance in transgenic rice

Kiwoung Yang [a], Sunyo Jung [b], Yonghyuk Lee [a], Kyoungwhan Back [a,*]

[a] *Department of Molecular Biotechnology, Biotechnology Research Institute, Chonnam National University, Gwangju 500-757, Republic of Korea*
[b] *School of Life Sciences and Biotechnology, Kyungpook National University, Daegu 702-701, Republic of Korea*

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Abstract

Protoporphyrinogen oxidase (Protox) of *Myxococcus xanthus* (*Mx* Protox) is a 49-kDa membrane protein that catalyzes conversion of protoporphyrinogen IX (Protogen IX) into protoporphyrin IX (Proto IX). Upon heterologous expression in transgenic rice plants, *Mx* Protox is dually targeted into plastids and mitochondria, increasing resistance against the herbicidal Protox inhibitor oxyfluorfen. Here, we describe the chemical synthesis of the *Mx* Protox gene by assembling several small synthetic DNA fragments derived by ligation-PCR. Codon usage in the resulting 1416-bp gene was modified to correspond to that of the *Arabidopsis* Protox gene, a change that resulted in a decrease in G+C content from 71 to 49%. The modified *Mx* Protox gene was used to generate transgenic rice plants *via Agrobacterium*-mediated transformation. Integration, expression, and inheritance of the transgenes were demonstrated by Southern, Northern, and Western blot analyses. In plants transformed with the modified, low G+C-content *Mx* Protox gene, levels of Protox expression and enzyme activity were low compared to the levels observed for plants transformed with the native *Mx* Protox gene. Nonetheless, like the native gene, the modified gene conferred a high level of resistance to the herbicide oxyfluorfen in a seedling growth test.

Keywords: Protoporphyrinogen oxidase; Transgenic rice; Peroxidizing herbicide; Synthetic gene; *Myxococcus xanthus*; G+C content

1. Introduction

Herbicides targeting the enzyme protoporphyrinogen IX (Protogen IX) oxidase (Protox) have been used commercially since 1960 to control annual grasses and dicotyledonous weeds in soybean (*Glycine max*), peanut (*Arachis hypogaea*), cotton (*Gossypium*), rice (*Oryza sativa*), and other crops [1–3]. Diphenyl ethers, *N*-aryltetrahydropthalimides, oxadiazon, and flumioxazin are herbicidal Protox inhibitors that, as partial structural analogs of Protogen IX, bind to and competitively inhibit Protox activity [4]. This inhibition prevents oxidation of Protogen IX into protoporphyrin IX (Proto IX, EC 1.3.3.4)[1] thus resulting in the accumulation of Protogen IX, which diffuses out of the chloroplasts to the cytoplasm. In the cytoplasm, Protogen IX is oxidized to Proto IX *via* peroxidase-like enzymes that are insensitive to Protox inhibitors. Cytoplasmic Proto IX readily interacts with oxygen in light to form singlet oxygen, causing peroxidation of membrane lipids and cell death [5,6].

In tobacco plants, there are two Protox isozymes sharing only 27% amino acid identity. One is expressed in chloroplasts and the other in mitochondria [7]. Genes for both Protox isozymes have played a pivotal role in the development of plants resistant to Protox-inhibiting herbicides [8–13].

In a previous report, we described the development of transgenic rice plants that overexpress a heterologous Protox gene from the bacterium *M. xanthus*. When this gene is expressed in transgenic rice plants, the *Mx* Protox protein is dually targeted into plastids and mitochondria *via* ambiguous transit signals, increasing resistance to oxyfluorfen, a

* Corresponding author. Fax: +82 62 530 2169.
E-mail address: kback@chonnam.ac.kr (K. Back).

[1] *Abbreviations used:*, Proto IX, protoporphyrin IX; Protogen IX, protoporphyrinogen IX; *Mx*, *Myxococcus xanthus*; Protox, protoporphyrinogen oxidase; G+C, guanosine + cytosine.

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herbicidal Protox inhibitor [14]. Overexpression of the *Mx* Protox transgene in rice confers a 200-fold level resistance to oxyfluorfen over the wild-type rice [9,14–16].

Mx Protox is a 49-kDa membrane protein that is strongly inhibited by the Protox-inhibiting herbicide acifluorfen [17]. However, transgenic rice plants expressing the *Mx* Protox showed a dramatically higher resistance against oxyfluorfen compared with the other transgenic rice plants expressing an *Arabidopsis* or *Bacillus subtilis* Protoxs [9,14,15]. Higher herbicide resistance in transgenic plants expressing *Mx* Protox is simply due to the overexpression of *Mx* Protox protein in both chloroplasts and mithochondria with 5- and 12-fold increase in Protox activity, respectively, over the wild-type rice [14].

Interestingly, the 71% G+C content of *Mx* Protox cDNA (GenBank Accession No. M73709) is considerably higher than the G+C contents of Protox cDNAs from *Arabidopsis* [18] or rice (GenBank Accession No. NM187739) (49 and 58%, respectively). The difference in codon usage between the *Mx* Protox gene and plant Protox gene make us suspect that the bacterial Protox gene might not be properly expressed in plants [19,20]. Therefore, we optimized the codon usage to more closely resemble that of plants without changing the *Mx* Protox amino acid sequence by synthesizing the full-length coding sequence for *Mx* Protox, and this synthetic gene was delivered into rice plants. Transgenic rice plants expressing the optimized *Mx* Protox gene had resistance to oxyfluorfen as high as rice plants expressing the native *Mx* Protox gene despite a lower level of Protox expression.

2. Materials and methods

2.1. Construction of a synthetic gene encoding M. xanthus Protox protein

Using plant-preferred codons [20], we designed a synthetic gene encoding the previously reported amino acid sequence for *Mx* Protox [17]. The codon usage was based primarily on that of the Protox gene from *Arabidopsis* (GenBank Accession No. D83139) because this *Arabidopsis* Protox has been utilized as a representative gene to develop many Protox inhibitor-resistant crops [10,11]. A total of six double-stranded *Mx* Protox gene fragments (A to F, Fig. 1B), ranging from 184 to 294 bp in length, were synthesized. These fragments contained overlapping, complementary DNA sequences of at least 12 bases and appropriate restriction enzyme sites to facilitate assembly and cloning of the gene. After synthesis, the fragments were ligated to generate a full-length, synthetic gene for *Mx* Protox.

For construction of double-stranded gene fragment A (253 bp in length), two different pairs of oligonucleotides, each 67–72 bases in length, were synthesized (Fig. 1C; Bioneer, Korea). Each pair (A_1/A_2 or A_3/A_4) possessed complementary regions of at least 12 bases. Complementary oligonucleotides (A_1 and A_2 or A_3 and A_4) were mixed at a concentration of 200 nM in 5 mM $MgCl_2$ containing 5 mM dNTPs and annealed by heating at 85 °C for 2 min, followed by gradual cooling to room temperature over 25 min. After annealing, the mixture was treated with DNA polymerase I (Invitrogen, USA) at 37 °C for 30 min to catalyze complementary-strand synthesis. The resulting mixtures were precipitated with ethanol and digested with *Xho*I restriction endonuclease.

To ligate the fragments, the digested A_1/A_2 and A_3/A_4 DNA fragment mixtures were combined and incubated in ligase buffer in the presence of T4 DNA ligase (5 U) (Invitrogen) at 21 °C for 90 min. Finally, the resulting synthetic DNA fragment A was amplified by PCR using the ligated DNA fragments as a template, 5′-d(ATCAAGCTT **ATG**CATCACATGCCAAGA)-3′ as the forward primer (AF; *Hin*dIII restriction site underlined; translation start codon in bold), and 5′-d(CGCGAATTCGTCCTTCAA GATTAAGAGCTGCTGCTAG)-3′ as the reverse primer (AR; *Eco*RI site underlined). The PCR product was digested with *Hin*dIII and *Xho*I, gel-purified, and ligated into pBluescript SK+ (Stratagene, USA). The integrity of the synthetic DNA insert was determined using the dideoxynucleotide chain termination method (Basic Science Research Center, Chonnam National University, Korea). Similar procedures were used for synthesis of DNA fragments B through F. The full-length synthetic Protox gene was assembled by ligating the *Hin*dIII/*Eco*RI gene fragment (A), *Eco*RI/*Pst*I gene fragment (B+C+D), and *Pst*I/*Kpn*I gene fragment (E+F) into the *Hin*dIII and *Kpn*I sites of pBluescript SK+ (Stratagene).

2.2. Isolation and analysis of nucleic acids

Total RNA was extracted using TRI REAGENT (Sigma, USA). Total RNA (10 μg) was fractionated on a formaldehyde-containing 1.0% agarose gel using 20 mM MOPS [3-(*N*-morpholino)-propanesulfonic acid] as the electrophoresis buffer. The gel was blotted onto a nylon membrane and hybridized with a synthetic DNA probe generated by PCR with the primer AF and the reverse primer 5′-d(GGGTACCCTACGGTGCATGAGAAGT)-3′ (carboxy-terminal sequence). Equal loading of RNA samples was confirmed by ethidium bromide staining of the gel prior to blotting.

Genomic DNA was isolated using DNAzol ES (Molecular Research Center, USA). Genomic DNA (5 μg) was digested with restriction enzymes, size-fractionated by electrophoresis on an 0.8% agarose gel, and blotted onto a nylon membrane (Nylon 66 plus, Pharmacia Biotech). Hybridizations were performed as previously described [21].

2.3. Bacterial expression studies and enzyme purification

An *Escherichia coli* expression system for synthetic Protox was developed using the T7 expression plasmid pET28(b) (Novagen, WI, USA). The full-length Protox gene was amplified by PCR using 5′-d(CATGCCA**TGG**A TCACATGCCAAGA)-3′ as the forward primer (*Nco*I site underlined; translation start codon in bold), 5′-d(GATA