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A tobacco plastidal transit sequence cannot override the dual targeting capacity of *Myxococcus xanthus* protoporphyrinogen oxidase in transgenic rice

S. Jung [a], Y. Lee [b], K. Back [b,*]

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

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Abstract

The effect of a plastidal transit sequence in *Myxococcus xanthus* protoporphyrinogen oxidase (Protox) on gene targeting ability was investigated by generating transgenic rice that overexpressed *M. xanthus* Protox with the additional plastidal transit sequence (TTS line). In transgenic lines TTS3 and TTS4, the Protox antibody cross-reacted with the mature *M. xanthus* Protox protein of 50 kDa. In an in vitro import system using the *M. xanthus* Protox gene with the plastidal transit sequence, *M. xanthus* protein was detected in both chloroplasts and mitochondria, confirming that it was targeted into both organelles, as in transgenic rice line, M4, that overexpressed *M. xanthus* Protox lacking the plastidal transit sequence. A prominent increase in chloroplastic and mitochondrial Protox activity was observed in TTS3 and TTS4 relative to the wild type. However, the increase was lower than that in transgenic line M4. Seeds from all transgenic lines (TTS3, TTS4, and M4) were able to germinate when treated with up to 500 μM of the Protox-inhibiting herbicide, oxyfluorfen, whereas seeds from the wild type failed to germinate even when treated at levels as low as 1 μM. After foliar application of oxyfluorfen, TTS3 and TTS4 exhibited a reduced Protox activity, however, it was much greater than uninhibited Protox activity of wild type. The great increase in conductivity was followed by the great accumulation of photodynamic protoporphyrin IX only in oxyfluorfen-treated wild-type plants, not in oxyfluorfen-treated TTS lines. The presence of the plastidal transit sequence neither excludes the intrinsic ability of subcellular translocation of *M. xanthus* Protox nor changes herbicide resistance in TTS lines.

Keywords: Protoporphyrin IX; Protoporphyrinogen oxidase; Herbicide resistance; Oxyfluorfen; Transgenic rice

1. Introduction

Tetrapyrrole biosynthesis is important in plants because it provides many essential molecules that are involved in light harvesting, energy transfer, signal transduction, detoxification, and systemic acquired resistance [1–3]. The last common step in the tetrapyrrole pathway to heme and chlorophyll is the oxidation of protoporphyrinogen IX (Protogen IX) to protoporphyrin IX (Proto IX)[1] [4,5], a reaction catalyzed by protoporphyrinogen oxidase (Protox, EC 1.3.3.4). Plants express two Protox isozymes: one is associated with the chloroplast envelope and thylakoid membranes [6], and the other is localized to mitochondria [7].

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

[1] *Abbreviations used:* ALAD, 5-aminolevulinic acid dehydratase; ALDH2, mitochondrial aldehyde dehydrogenase; Proto IX, protoporphyrin IX; Protogen IX, protoporphyrinogen IX; Protox, protoporphyrinogen oxidase; RbcS, chloroplast small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase; TMP, *Myxococcus xanthus* Protox with a plastidal transit sequence.

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There is increased interest in the mechanisms of plant chlorophyll and heme synthesis owing to recent studies describing the light-dependent action of photobleaching diphenylether herbicides that inhibit Protox [8–12]. Following Protox inhibition, the accumulated substrate, Protogen IX, diffuses out of the plastids and is rapidly oxidized to Proto IX at the plasma membrane by herbicide-insensitive, non-specific Protox-like activity [13,14]. The Proto IX produced in the cytoplasm cannot be consumed by the porphyrin-synthetic pathway because Mg-chelatase and Fe-chelatase, which use Proto IX as a substrate, are located only in chloroplasts and mitochondria. Cytoplasmic Proto(gen) IX, a potent photosensitizer, absorbs light energy that is used in detrimental reactions in which energy or electrons are subsequently transferred to oxygen, resulting in the formation of highly reactive oxygen species, rapid lipid peroxidation, and cell death.

A large number of Protox genes from prokaryotes and eukaryotes have been cloned. Almost all eukaryotic Protoxes, with the exception of a spinach Protox II, feature single targeting proteins that are destined to be expressed in one subcellular site in either chloroplasts or mitochondria [15]. In tobacco (*Nicotiana tabaccum*), two forms of Protox that share only 27% amino acid identity are independently targeted to two different cellular compartments: chloroplasts and mitochondria [16]. In contrast, mammals possess only a mitochondrial form of Protox that is imported into the inner mitochondrial membrane [17,18]. Several strategies have been used to produce peroxidizing-herbicide-resistant transgenic plants involving the plastidal overexpression of Protox genes from *Arabidopsis thaliana* [19,20]. The overproduction of Protox in the chloroplasts of transgenic plants neutralizes the action of Protox-inhibiting herbicides. For example, transgenic maize overexpressing the *Arabidopsis* Protox mutant gene showed a 1000-fold higher level of resistance to the Protox-inhibiting herbicide butafenacil [12,21]. In addition, a study of soybean cell lines showed that oxyfluorfen-resistant cells had a 9-fold higher level of mitochondrial Protox activity than did non-resistant cells [22], suggesting a key role of mitochondrial Protox in herbicide resistance.

In particular, a *Myxococcus xanthus* Protox gene, without an additional plastidal transit sequence, is expressed in both the chloroplasts and mitochondria of rice, and corresponding transgenic plants display dramatically increased herbicide resistance [23]. The mechanism by which *M. xanthus* Protox partitions into chloroplasts and mitochondria may be associated with an ambiguous N-terminal sequence of *M. xanthus* Protox. In the present study, in vivo transgenic analyses and in vitro translocation assays were performed to determine whether the introduction of plastidal transit sequence to the *M. xanthus* Protox gene could influence its dual targeting ability of *M. xanthus* Protox, and thus, influence herbicide resistance. The tobacco plastidal transit sequence could have three possible effects on the targeting capacity of *M. xanthus* Protox. First, the tobacco transit sequence could completely override the unknown targeting signal sequences in Protox. Second, the transit sequence could increase chloroplast targeting by Protox, but not change mitochondrial targeting. Finally, the transit sequence could have no effect at all on the dual targeting capacity of Protox. In this paper, we showed that the transit sequence affected neither the intrinsic dual targeting capacity nor the enhanced targeting into chloroplasts over mitochondria. It seems likely that the transit sequence reduced chloroplast targeting by interfering with the chloroplast targeting capacity. The herbicide resistance of resulting transgenic rice plants were further compared with wild-type rice and transgenic rice overexpressing a *M. xanthus* Protox lacking the plastidal transit sequence.

2. Materials and methods

2.1. Construction of the binary vector and rice transformation

Agrobacterium-mediated transformation was used to generate transgenic rice plants. Scutellum-derived rice calli (*Oryza sativa* cv. Dongjin) were co-cultured with *Agrobacterium tumefaciens* LBA4404 harboring pGA1611:TMP binary vector (Fig. 1). A plastidal transit sequence encoding a 63 amino acid protein was obtained from the tobacco plastidal Protox gene and exhibited a typical plastidal transit sequence, i.e., rich in Ser/Thr and deficient in Asp/Glu/Tyr which was shown to translocate the passenger protein into chloroplasts [24]. The transit sequence was prepared as a *Hin*dIII–*Sac*I fragment. The complete open reading frame of *M. xanthus* Protox was amplified using the primers 5′-GCTGGAGCTCATGCACCACATGCCGAGG-3′ (*Sac*I site underlined) and 5′-GGTGGTTAACTACGGGGCG TGGGAGGT-3′ (*Hpa*I site underlined). Both DNA fragments encoding the transit sequence and *M. xanthus* Protox were double-ligated into pBluescript-SK (Stratagene, La Jolla, CA, USA), predigested with *Hin*dIII and *Sma*I. After verifying the sequence integrity, the *Hin*dIII–*Hpa*I fragments of TMP were purified and ligated into the same restriction sites of vector pGA1611, between the maize ubiquitin promoter and the nos 3′ terminator. The resulting pGA1611:TMP was then transformed into *A. tumefaciens* LBA4404; the *A. tumefaciens*-mediated transformation was carried out as previously described [24]. The *M. xanthus* Protox gene was provided by Dr. H.A. Dailey (University of Georgia).

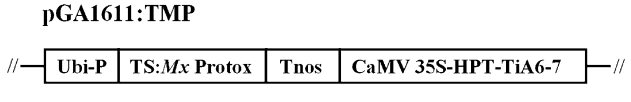

Fig. 1. Schematic of T-DNA of the binary vector used for plant transformation. The pGA1611:TMP was used for the transformation of rice calli. Ubi-P, maize ubiquitin promoter; TS, tobacco plastidal transit sequence; *Mx* Protox, *Myxococcus xanthus* protoporphyrinogen oxidase; Tnos, nopaline synthase terminator; CaMV 35S, cauliflower mosaic virus 35S promoter; HPT, hygromycin phosphotransferase; TiA6-7, TiA6-7 terminator.