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Co-expression of a NADPH:P450 reductase enhances CYP71A10-dependent phenylurea metabolism in tobacco

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Abstract

A soybean cytochrome P450 monooxygenase, designated CYP71A10, catalyzes the oxidative N-demethylation or ring methyl hydroxylation of a variety of phenylurea herbicides. The ectopic expression of CYP71A10 in tobacco was previously shown to be an effective means of enhancing whole plant tolerance to the compounds linuron and chlortoluron. Because P450 enzymes require ancillary proteins to catalyze the transfer of electrons from NADPH to the functional heme group of the P450, it is possible that the endogenous levels of these companion proteins may be insufficient to support the optimal activation of a highly expressed recombinant P450. In the present report, we have generated transgenic tobacco that simultaneously express CYP71A10 and a soybean P450 reductase. Transformed plants that express both CYP71A10 and the P450 reductase demonstrated 20–23% higher metabolic activity against phenylurea herbicides than control plants expressing CYP71A10 alone. These results suggest that herbicide tolerance strategies based on the expression of P450 genes may require concomitant expression of a cognate electron transport partner to fully exploit the herbicide metabolic capacity of the P450.

1. Introduction

Cytochromes P450 (P450s) are multifunctional enzymes that catalyze diverse metabolic processes including the detoxification of xenobiotics [1]. P450s function by oxidizing their substrates with one atom of molecular oxygen and reducing the other atom of oxygen to water using NADPH as an electron donor. The microsomal P450 redox system found in plants is termed a Class II system, whereby electron transfer between NADPH and the P450 is mediated either by an FAD- and FMN-containing NADPH:P450 reductase (CPR)[1] or by a FAD-containing cytochrome b_5 reductase and a cytochrome b_5 (cyt b_5) [2]. The functional dependence of specific P450s on cyt b_5 was demonstrated by experiments showing that inactivation of cyt b_5 by transposon insertion reduced the activity of petunia flavonoid 3′,5′ hydroxylase [3]. In contrast, bacterial and mitochondrial P450

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[1] *Abbreviations used:* CPR, NADPH:P450 reductase; Cyt b_5, cytochrome b_5; GmCPR, soybean NADPH:P450 reductase.

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redox systems are designated Class I systems, where the electron transport chain between NADH (in bacteria) or NADPH (in mitochondria) and the P450 includes a FAD-containing reductase and an iron–sulfur redoxin. Class III P450 systems such as allene oxide synthase (CYP74), tromboxane synthase (CYP5A1) and prostacylin synthase (CYP8) contain no auxiliary redox partners. Because P450 isozymes of the Class I and II systems require two electrons to complete their catalytic cycles, the rate of electron transfer between the various redox partners is an important determinant of P450 activity [2].

The mechanistic details of the P450 catalytic cycle have been reviewed by White and Coon [4]. Briefly, the substrate is bound to ferric P450, followed by P450 reduction to the ferrous state by an electron transferred from CPR. Following the first reduction step, molecular oxygen is bound to ferrous P450 and a second electron is transferred from either CPR or cyt b_5. The second reduction of ferrous P450 elicits the splitting of the oxygen–oxygen bond of dioxygen, and one of the two oxygen atoms is reduced to water. The other oxygen atom is incorporated into the substrate that, in turn, is released as the corresponding alcohol restoring the ferric state of P450 and completing the cycle. The rate-limiting step of the P450 catalytic cycle is isoenzyme-specific and may be at the level of substrate binding, reduction efficiency or substrate release [5].

The reduction rate in a Class II P450 redox system is a function of several parameters, such as the distance, the association constant, and the redox potential difference between the P450 and the CPR, in addition to the dielectric constant of the medium and the concentration of the respective redox partners [6]. As a result, the ratio and the "compatibility" between P450 redox components can have dramatic effects on P450 activity. For example, in yeast heterologous expression of plant P450s can be significantly enhanced by simultaneously overexpressing the yeast CPR, suggesting that native yeast CPR concentrations are insufficient for optimal expression of plant P450 cDNAs [7,8]. In studies conducted using reconstituted P450 systems, the molar ratio between P450 and CPR had to be optimized to achieve maximal substrate turnover [9–11]. The activities of heterologously expressed plant P450s in yeast are strongly influenced by the selection of the redox partner. CYP76B1 and CYP81B1 isolated from Jerusalem artichoke showed higher metabolic activities in the presence of an Arabidopsis CPR, ATR1, than in the presence of a yeast CPR [12,13]. In addition, one of the isoforms of CYP81B1, CYP81B1l, showed higher capric acid hydroxylase activity in yeast overexpressing ATR2, while the other isoform, CYP81B1s, catalyzed the same reaction more efficiently in the presence of ATR1 [13]. Another experiment showed that ATR1 supported a rabbit P450-mediated O-deethylation of ethoxycoumarin more efficiently than HSR1, a human CPR, indicating that the source of an optimal P450 redox partner may be of phylogenetically distant origin [14]. In animal systems, CPR concentrations in liver microsomes were reported to be inadequately low to support the electron requirement of P450 isoenzymes and thus represented a limiting factor in P450 metabolic activities [15]. Although the above studies suggest that CPR levels can be rate-limiting in P450-mediated reactions in animals and yeast, there is currently a paucity of information pertaining to potential limitations of plant CPRs.

We have recently demonstrated that the gene encoding a soybean P450 designated CYP71A10 can serve as an effective tool in conferring herbicide tolerance in transgenic plants. Tobacco plants, transformed with CYP71A10, rapidly metabolized the phenylurea herbicides fluometuron, linuron, chlortoluron, and diuron [16]. The transgenic plants showed a 16-fold increase in tolerance to linuron, when the herbicide was pre-transplant incorporated, and a 12-fold increase in tolerance to postemergence applications [17]. Similarly, a P450 gene, isolated from Jerusalem artichoke (CYP76B1), was also shown to be effective in mediating herbicide resistance, conferring a 20-fold increase in linuron tolerance in transgenic tobacco and Arabidopsis plants [18].

To exploit the full potential of plant P450 genes as herbicide tolerance agents, it is necessary to investigate and establish the specific redox environments needed to facilitate optimal P450 enzyme activity. There are two primary strategies that can

be implemented to enhance the requisite cellular CPR levels: (1) generate and express a translational fusion between a P450 and a CPR gene; or (2) independently co-express the P450 and CPR genes on separate constructs. When Didierjean et al. [18] tested fusion constructs that joined the linuron-detoxifying CYP76B1 gene to a Jerusalem artichoke CPR, less herbicide metabolism was observed than with transgenic plants that only expressed CYP76B1. Immunoblot analysis suggested that the CYP76B1:CPR fusion protein was unstable in the plant. The primary objective of the present study was to test whether the co-expression of a soybean CPR (GmCPR) can enhance the CYP71A10-dependent phenylurea metabolism of phenylurea herbicides in tobacco. Our results support the conclusion that endogenous CPR levels in plants can limit the herbicide metabolic activity of a heterologous P450 and that co-expressing a CPR represents an effective strategy for overcoming this limitation.

2. Materials and methods

2.1. Isolation of the GmCPR cDNA

An Arabidopsis CPR cDNA was identified in a expressed sequence tag (EST) database and obtained from the Arabidopsis Biological Resource Center at Ohio State University. Several candidate GmCPR cDNAs were isolated from a soybean leaf cDNA library cloned into the Lambda ZAP II cloning vector (Statagene) using the Arabidopsis CPR cDNA as a hybridization probe. Positive clones were analyzed by gel electrophoresis and the largest cDNA was subjected to DNA sequence analysis. Homology to other plant CPR sequences deposited in GenBank was verified using the BLAST algorithm [19].

2.2. Tobacco transformation with GmCPR

A full-length GmCPR cDNA was placed under the transcriptional regulation of the cauliflower mosaic virus 35S promoter by replacing the GUS cDNA of the pBI121 binary expression vector (Clontech, Palo Alto, CA, USA) with GmCPR. The resulting construct was used to transform Agrobacterium strain LBA 4404. Agrobacterium-mediated transformation of *Nicotina tobacum* SR1 tobacco leaf disks, tissue culturing and plant regeneration were performed as previously described by Horsch et al. [20].

2.3. Generation of anti-CYP71A10 antibodies

A 500-bp-long fragment of the CYP71A10 open reading frame, located at the 3′ end of the CYP71A10 cDNA encoding the C-terminal 131 amino acids of the P450, was cloned into a His-tag containing pRSET vector (Invitrogen, Carlsbad, CA, USA) and expressed in Escherichia coli. The His-tagged fusion protein was purified using a nickel-chelating resin column following the protocols specified by the manufacturer (Invitrogen). Anti-CYP71A10 polyclonal antibodies were raised in a rabbit by Covance Research Products Inc (Princeton, NJ, USA).

2.4. Immunoblot analysis of GmCPR and CYP71A10

Protein extracts were prepared by homogenizing 5 g leaf tissue of 6-weeks-old tobacco in 40 ml of extraction buffer [50 mM Hepes (pH 7.5), 250 mM sucrose, 15 mM mercaptoethanol, 2.5% (wt/vol) PVPP] with a tissue homogenizer. The leaf extracts were filtered through two layers of Miracloth (CalBiochem, LaJolla, CA, USA) and centrifuged for 20 min at 10,000*g*. Microsomal fractions were recovered by centrifugation of the supernatant for 1 h at 100,000*g*. The microsomal pellet was resuspended in buffer [50 mM Hepes (pH 7.5), 30% (vol/vol) glycerol] and stored at −80 °C. Protein concentrations were determined as described by Bradford [21]. For immunoblot assays, 15 μg of microsomal protein was separated using SDS–PAGE. Proteins were transferred onto PVDF membranes (Amersham Biosciences, Piscataway, NJ, USA) and incubated overnight in a 1:3000 dilution of polyclonal antibody prepared against either Jerusalem artichoke CPR or CYP71A10. Immunoreactive bands were visualized using an anti-rabbit HRP-conjugated secondary antibody and the ECL Western Blotting Detection System (Amersham).

2.5. Generation of the CYP71A10/GmCPR and CYP71A10/pBI tobacco lines

A nonsegregating CYP71A10-transformed line, designated 25/2 [16], and several primary transformants carrying the GmCPR transgene or the pBI121 control plasmid were grown in a greenhouse. GmCPR-transformed lines displaying high levels of transgene expression (as determined by immunoblot analysis) were crossed with individuals of the 25/2 or pBI121 lines.

2.6. Herbicide tolerance

F_1 generation seeds from CYP71A10/GmCPR-transformed tobacco and CYP71A10/pBI-transformed control plants were placed onto Petri dishes containing MS salts and a commercial formulation of linuron, LOROX 50 DF, at active ingredient concentrations ranging from 2.5 to 4.5 μM. The Petri dishes were incubated at 27 °C with a 16/8 h light/dark cycle. Phytotoxic effects were determined visually by comparison to control plants and plants grown in the absence of the herbicide.

2.7. Herbicide metabolism assays

Single leaves (approximately 0.3 g) of control and CYP71A10/GmCPR tobacco plants were excised and their petioles inserted into 100 μl of H_2O containing radiolabeled linuron, fluometuron or chlortoluron. The leaves were placed in a growth chamber maintained at a temperature of 27 °C and incubated until the entire volume of the herbicide solution was drawn up by the transpirational stream (approximately 3 h). The leaves were subsequently transferred into an Eppendorf tube containing distilled water and incubated for an additional 4 h.

^{14}C-labeled herbicide was extracted from the leaves by grinding manually for 5 min in 150 μl methanol with a plastic pellet pestle. Approximately 70% of the absorbed herbicide was extracted in the methanol-soluble fraction and an additional 26% was recovered by combusting the methanol-insoluble fraction in a biological materials oxidizer (Model OX-500, R.J. Harvey Instrument, Hillsdale, NJ). After centrifugation for 3 min at 14,000*g*, 75 μl of the supernatant was analyzed by TLC. Samples were spotted onto 250 μm Whatman K6F silica plates and developed in a benzene/acetone 2:1 (v/v) solvent system. The developed plates were scanned with a Bioscan System 400 imaging scanner. Substrate conversion was quantified based on the ratios of the parent compound and the produced metabolites determined from the TLC profiles.

2.8. Statistical analysis

Potted plants were arranged in a completely randomized design using three independently transformed tobacco lines for each phenotype. From each transformant line, five individuals were tested. Herbicide conversion data was subjected to square-root transformation to stabilize variance. Inferences concerning significant and nonsignificant differences were derived from the ANOVA of the transformed data.

3. Results

To determine whether the overexpression of CPR activity can enhance the rate of herbicide catabolism in CYP71A10-transformed tobacco, we initially isolated a CPR cDNA from soybean (GmCPR).[2] Because compatibility between the redox partners is a prerequisite for efficient electron transport, soybean was considered to be the optimal source for obtaining a compatible electron donor for the soybean-derived CYP71A10 enzyme. Nucleotide sequence analysis revealed that the GmCPR cDNA contained an open reading frame capable of encoding a 689 amino acid protein with a predicted molecular mass of 76.5 kDa. The predicted amino acid sequence of GmCPR was 80% identical to that of mungbean CPR and contained a flavodoxin, an FAD- and a NADP-binding domain, features that are shared by CPRs (data not shown).

[2] GeneBank accession number for GmCPR is AY170374.

To facilitate the expression of CPR activity in transgenic plants, GmCPR was cloned downstream of the strong, constitutive 35S promoter of cauliflower mosaic virus. Several independent transgenic tobacco plants possessing the 35S:GmCPR construct were screened by immunoblot analysis using antibodies generated against a Jerusalem artichoke CPR [22]. Transgenic lines that displayed substantially greater CPR accumulation than wild-type control tobacco plants were selected for further study (data not shown). CYP71A10/GmCPR tobacco lines (carrying both the CYP71A10 and GmCPR transgenes) were generated by crossing a homozygous CYP71A10 line [17] with ten T_0 GmCPR lines that showed high levels of transgene expression. Because the resulting F_1 populations were segregating for the GmCPR transgene, immunoblot analyses were performed to identify CYP71A10/GmCPR individuals in which GmCPR levels were greater than those in the CYP71A10/pBI control plants (Fig. 1). Consistent with the results reported by Shet et al. [23], three immunoreactive bands were detected in the immunoblot analysis of tobacco microsomes with anti-CPR antibody. Because CYP71A10/GmCPR F_1 individuals possess only half the CYP71A10 copies as the parental CYP71A10 line, crosses were also made with a control tobacco plant transformed only with the pBI121 vector to generate F_1 plants (CYP71A10/pBI) that would enable a fair comparison of CYP71A10-mediated herbicide metabolic activity in the presence or absence of increased CPR activity.

To measure the effect of GmCPR overexpression on the herbicide catalytic activity of wild- type tobacco plants and plants transformed with CYP71A10, the rate of phenylurea herbicide metabolism was compared between wild type, GmCPR, CYP71A10/pBI, and CYP71A10/GmCPR plants. As shown in Table 1, a basal rate of approximately 25 pmol herbicide metabolized per hour is observed for the compounds linuron and fluometuron in wild-type tobacco plants. Chlortoluron is metabolized at nearly a 3-fold higher rate. These results are consistent with our previous observations [16]. The expression of GmCPR in tobacco plants did not significantly alter the basal rate of herbicide metabolism for any of the three phenylurea compounds.

Consistent with our previous reports [16,17], tobacco plants transformed with CYP71A10/pBI metabolized all three herbicides at a much faster rate than wild type plants (Table 1). Co-expression of GmCPR enhanced the herbicide metabolism even further. Over an 8-h incubation period, CYP71A10/GmCPR plants metabolized approximately 20% more herbicide than CYP71A10/pBI individuals for each compound tested. Although the CYP71A10/GmCPR and CYP71A10/pBI plants are expected to possess a quantitatively and qualitatively equivalent complement of CYP71A10 transgenes, we conducted an immunoblot analysis of the respective transgenic plants using anti-CYP71A10 antibody to test whether the two F_1 lines accumulated similar levels of CYP71A10 enzyme. As shown in Fig. 2, CYP71A10/GmCPR and CYP71A10/pBI individuals appeared to accumulate the soybean P450 enzyme in similar abundance.

When presented with linuron as a substrate, CYP71A10 typically generates two metabolic products: *N*-demethyl linuron and *N*-demethoxy

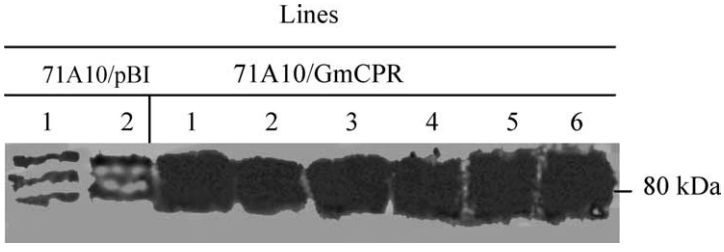

Fig. 1. Immunodetection of CPR in tobacco leaf extracts isolated from two control (1–2) and six 71A10/GmCPR (1–6) transgenic lines using the anti-Jerusalem artichoke CPR antibody.

Table 1
Herbicide metabolism by excised leaves of transgenic and wild-type tobacco

Herbicide	Line #	Herbicide metabolized (pmol/h)[A,B]			
		Wild-type	GmCPR	71A10/pBI	71A10/GmCPR
Linuron	1	25	26	152	190
	2	22	25	149	179
	3	23	24	156	181
	Mean	23a	25a	152b	183c
Fluometuron	1	24	24	162	192
	2	21	27	168	199
	3	29	28	165	204
	Mean	25a	26a	171b	198c
Chlortoluron	1	69	73	141	187
	2	63	77	144	176
	3	75	65	148	168
	Mean	69a	72a	144b	177c

[A] An equal amount of 1.5 nmol herbicide was added for each experiment.
[B] Means in the same row followed by the same letter are not significantly different at $\alpha = 0.05$, according to Fisher's protected LSD test.

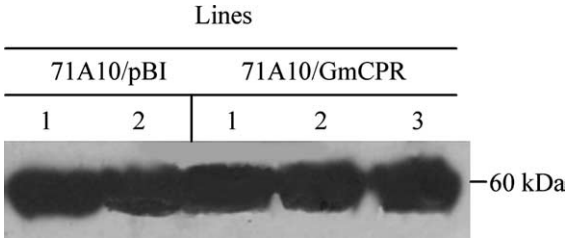

Fig. 2. Immunodetection of CYP71A10 in tobacco leaf extracts isolated from two control (1–2) and three 71A10/GmCPR (1–3) lines.

linuron [16]. To determine whether effects of GmCPR are solely quantitative (affecting only the rate of the reaction) versus qualitative in nature (also affecting the relative ratios of the end products), chromatographic profiles of ^{14}C-linuron treated tobacco leaves were examined and the individual products were quantified. A typical radiochromatogram is shown in Fig. 3. Peaks corresponding to *N*-demethyl linuron (M_1), an intermediate product of linuron N-demethylation (M_2), and *N*-demethoxy linuron (M_3) were observed with both CYP71A10/GmCPR and CYP71A10/pBI plants. Consistent with the results presented in Table 1, a significant difference in the ratio of unmetabolized parental compound to total metabolic product ($M_1 + M_2 + M_3$) was

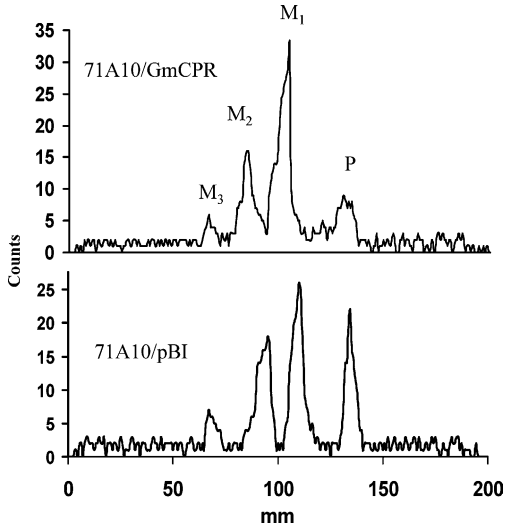

Fig. 3. Thin-layer radiochromatograms of leaf extracts isolated from 71A10/GmCPR and 71A10/pBI tobacco following a 8-h incubation with ^{14}C-linuron. M_1: *N*-demethyl linuron; M_2: intermediate; M_3: *N*-demethoxy linuron; P: parent.

observed between CYP71A10/GmCPR and CYP71A10/pBI individuals. However, no significant difference was observed among the relative ratios of the three metabolites between the two F_1 genotypes (data not shown).

Although co-expression of GmCPR significantly increased the rate of CYP71A10-mediated

herbicide metabolism in transgenic plants, no differences were observed between linuron treated CYP71A10/GmCPR and CYP71A10/pBI individuals at the whole plant level (data not shown). This was not unexpected, however, given the observation that the major reaction product, *N*-demethyl linuron, retains some herbicidal activity [24]. Therefore, in the case of plants transformed with CYP71A10, the maximal tolerance attainable appears to be ultimately defined by the sensitivity of the plant to *N*-demethyl linuron.

4. Discussion

To investigate the effect of GmCPR overexpression on CYP71A10- and native P450-mediated phenylurea herbicide metabolism, we measured the rate of ^{14}C-fluometuron, ^{14}C-linuron, and ^{14}C-chlortoluron metabolism in detached leaves of GmCPR, CYP71A10/GmCPR, and appropriate control tobacco plants. Our results demonstrated that GmCPR overexpression significantly enhanced CYP71A10-mediated herbicide metabolism in CYP71A10/GmCPR tobacco, suggesting that the endogenous levels of CPR in tobacco are unable to support maximal activation of the soybean P450. The increased rate of phenylurea metabolism in CYP71A10/GmCPR tobacco could alternatively be explained if compatibility between CYP71A10 and GmCPR were greater than that between CYP71A10 and tobacco CPR. Several studies showed that the catalytic efficiency of P450 isozymes was dependent upon the origin of its redox partner (see Section 1). Inconsistent with this scenario, however, are the observations that the ratio of CPR genes to P450 genes in plants is very low. In Arabidopsis, for example, 246 distinct P450 genes have been identified in the genome, compared to only two CPR genes [25]. The low isozymal diversity and high amino acid sequence homology among plant CPRs also suggest a high degree of interspecific compatibility between CPR and P450 isozymes.

The absence of an increase in herbicide metabolism in the GmCPR plants could indicate that phenylurea metabolism in tobacco is predominantly catalyzed by enzymes other than P450s. Although the involvement of plant P450s in phenylurea metabolism has been demonstrated in certain plant species [26–28], other hemeproteins, such as chloroperoxidases [29], peroxidases [30], and lipoxygenases [31] have also been shown to exhibit *N*-demethylase activity.

An additional explanation may be that electron transport for native, P450-dependent phenylurea metabolism is mediated by CPR in GmCPR plants, but the concentration of CPR is not rate-limiting in the absence of a P450 transgene that is driven by a strong promoter and/or exhibits a high turnover number. Unlike GmCPR plants, CYP71A10/GmCPR tobacco demonstrated an enhanced rate of herbicide metabolism relative to the corresponding CYP71A10/pBI control suggesting that either CPR concentrations are rate-limiting in the presence of a strong promoter-controlled P450 transgene or CYP71A10 is more compatible with GmCPR than with native tobacco CPRs. The role of CYP71A10 in phenylurea herbicide metabolism has been described [16], but the native function of the gene and endogenous substrates of the enzyme remains unknown. Therefore, it is conceivable that the increased demand for CPR-mediated electron transport in CYP71A10 plants may not be a result of elevated phenylurea metabolism alone but also a consequence of increased electron consumption arising from all CYP71A10-catalyzed reactions including the metabolism of endogenous as yet unknown substrates. We have previously isolated two P450 cDNAs from cotton that showed 68 and 75% amino acid sequence identity with CYP71A10 indicating that homologs of CYP71A10 exist in other plant species (unpublished data).

Our results, that endogenous CPR levels appear to become limiting when overexpressing CYP71A10, can have important ramifications for any transgenic application of plant P450s regardless of whether the objective is to provide herbicide tolarance or any other useful trait. If the products of P450 transgenes can compete with endogenous P450 isozymes for limited CPR pools, vital cellular functions may be compromised. Such competition may interfere with important biosynthetic processes and reduce the agronomic performance of the commodity. In applications designed to

achieve maximal expression of a foreign P450 in transgenic plants, without negatively impacting the function of essential endogenous P450s, simultaneous expression of a CPR gene may prove to be a necessity.

CPR and P450 genes can be simultaneously introduced into transgenic plants using one of two formats. In one approach, a translational fusion is constructed between the P450 and the CPR [18,32,33], and in the other strategy, the two transgene constructs are introduced independently as illustrated in our experiments. Although both approaches have the potential of producing functional proteins, a recent study reported that a translational fusion protein between a Jerusalem artichoke CPR and CYP76B1 exhibited lower activity towards phenylurea herbicides than CYP76B1 expression alone [18]. The authors speculated that the low activity of the fusion protein was a result of low protein expression, reduced stability or insufficient rate of electron transfer to CYP76B1. They also proposed that the connecting segments of the two peptides needed to be optimized to obtain stable and catalytically active products. These results, therefore, suggest that constructing a linkage between a CPR and a P450 is not a trivial process and revealed some of the technical difficulties that are potentially associated with the fusion protein approach. In contrast, the independent expression of GmCPR and CYP71A10 using two separate constructs is a straightforward strategy that proved to increase P450 activities in our experiments. We believe that this approach deserves consideration in future efforts directed to engineer P450-mediated processes.

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