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Functional characterization of NADPH-cytochrome P450 reductase and cinnamic acid 4-hydroxylase encoding genes from Scoparia dulcis L.

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Introduction

In contrast, it has been reported that higher plants have one to three copies of CPR (Rana et al. 2013). In addition, Ro et al. (Ro et al. 2002) classified CPRs into two classes, class I and class II, based on N-termi- nal sequences. CPRs classified in class I have a short N-terminal sequence and are constitutively expressed in plants, whereas class II CPRs are expressed in response to stress or elicitors/injury.

Scoparia dulcis L. (Plantaginaceae) is a perennial herb widely distributed in tropical areas. Several unique dit- erpenes (ex, scopadulcic acid B [SDB] and scopadulciol etc.) have been isolated from S. dulcis and have been shown to have various biological activities (Hayashi 2000). Hayashi et al. demonstrated that the biosynthe- sis of SDB is markedly activated in Scoparia leaf tissues by treatment with methyl jasmonate (MJ) (Hayashi et al. 1999; Nkembo et al. 2006). We recently discovered novel candidate genes (encoding diterpene synthase and P450) potentially involved in SDB biosynthesis by transcrip- tome analysis (Yamamura et al. 2017).

P450s are membrane proteins that requires a redox partner for expression of their enzyme activity; there- fore, preparation of recombinant P450 proteins has been mainly performed in eukaryotic expression systems, such as yeast (Yamamura et al. 2001; Hayashi et al. 2007) and insect cells (Ohnishi et al. 2012). Recently, several strate- gies have been developed for the expression of eukaryotic P450s in Escherichia coli (prokaryotic expression system) to characterize their activities (Hausjell et al. 2018). This approach may be applied to elucidate the various biosyn- thetic mechanisms of secondary metabolism in higher plants.

In this study, we isolated a CPR essential for the activ- ity of P450 from S. dulcis.

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The isolated Scoparia CPR (SdCPR) was further characterized for a monooxygenase activity with Scoparia cinnamic acid 4-hydroxylase P450 (SdC4H; CYP73A111) in vitro. The expression patterns of SdCPR and the SdC4H were also examined in Scoparia leaves after treatment with elicitor and wounding.

Materials and methods

Plant material and treatments

Scoparia dulcis L. were grown in sterile conditions on half-strength Murashige and Skoog plates at 25 °C in continuous light. Eight-week-old plants were used for all experiments. All plant leaves were evenly sprayed (three times) with 0.1 mM aqueous solutions of MJ (Sigma- Aldrich, MO, USA) and salicylic acid (SA; Nacalai Tesque, Kyoto, Japan), which were pre-dissolved in 99% ethanol. After incubation for 0–6 h, the second leaves from the top (fully expanded leaf) were used for qPCR. For mechanical wounding treatment, second leaves were cut into 2-mm fragments and incubated for 1–8 h in a petri dish (floated on 10 mL distilled water containing 0.005% (w/v) chloramphenicol). Samples were collected and frozen immediately in liquid nitrogen and stored at − 80 °C.

Cloning of SdCPR and SdC4H genes

Total RNA was extracted from S. dulcis leaves using TRI- zol (Thermo Fisher Scientific, Waltham, MA, USA) in accordance with the manufacturer's instructions. First strand cDNA was synthesized using a PrimeScript II 1st strand cDNA Synthesis Kit (Takara Bio Inc., Kusatsu, Shiga, Japan). cDNAs of SdCPR and SdC4H were isolated using degenerate primers (Additional file 1: Table S1). 5′- and 3′-end amplifications were carried out using a 5′ and 3′ rapid amplification of cDNA ends Kit, 2nd Genera- tion (Roche Diagnostics GmbH, Mannheim, Germany) in accordance with the manufacturer's instructions. The PCR products were subcloned into a pGEM-T easy vec- tor (Promega, Madison, WI, USA). All DNA sequences of PCR-amplified open reading frames (ORFs) were con- firmed using an ABI 3130 Genetic Analyzer (Applied Biosystems, Waltham, MA, USA).

Heterologous expression of SdCPR and SdC4H in E. coli

The ORFs of SdCPR and SdC4H were amplified using Pwo DNA polymerase (Roche). The PCR products were inserted into the expression vector pET28b (Merck Mil- lipore,

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Burlington, MA, USA) using an In-fusion HD Cloning Kit (Takara Bio Inc.). E. coli BL21 (DE3) cells harboring the expression vector were grown overnight in LB medium with 50 μg mL−1 kanamycin and 1% glu- cose at 37 °C in a shaking incubator, then diluted 1:25

into fresh LB medium supplemented with 50 μg mL−1 kanamycin. Cells were grown at 37 °C at 200 rpm until absorbance at 600 nm reached 0.4 − 0.6, and then 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was

added. The culture was shaking at 200 rpm at 25 °C over- night for protein expression. The bacterial cells were col- lected by centrifugation at 3000 rpm for 5 min at 4 °C and washed twice with 4 °C wash buffer (10 mM Tris–HCl [pH 7.5], 150 mM NaCl). Then, the washed cell pellet was suspended in the BugBuster Protein Extraction Reagent (Novagen-Merck Millipore) and His-tag recombinant proteins were purified from the supernatant using Mag- neHis Ni-Particles (Promega) with elution buffer con- taining 1 M imidazole.

Recombinant enzyme assays

The activities of SdCPR was assayed as described by Yang et al. (Yang et al. 2010). The assay was performed in a Hitachi U-2000A UV spectrophotometer, and reduction of cytochrome c was monitored by the increase in absorbance at 550 nm, at 25 °C, in 50 mM Tris buffer, pH 7.4, con- taining 100 μM cytochrome c and 100 μM NADPH. The reaction was started by the addition of NADPH. A molar absorption coefficient of 21 mM−1 cm−1 for cytochrome c was used for quantification. Reduction of dichloro- phenol indophenol (DCPIP) was monitored at 600 nm (20.6 mM−1 cm−1), ferricyanide ($K_3Fe(CN)_6$) at 424 nm (1.02 mM−1 cm−1). To determine kinetic the param- eters for cytochrome c, 100 μM NADPH was added to the reaction mixtures containing varying concentrations of cytochrome c. The kinetic parameters for NADPH were measured using 100 μM cytochrome c with varying NADPH concentrations. The substrate concentration for

half maximal activity (K_m) and maximum rate of reaction (V_{max}) values were obtained using Hanes–Woolf plot anal- ysis. In vitro C4H enzyme assays were initiated by adding 2 mM NADPH to the reaction mixture (1 mL total volume) containing 50 mM phosphate buffer (pH 7.4), 1 mM trans- cinnamic acid (Wako, Osaka, Japan), 50 μg recombinant

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SdCPR soluble fraction, and 100 µg recombinant SdC4H soluble fraction. After incubation at 30 °C for 30 min, the reaction was stopped by adding 67 µL 6 M HCl, and the reaction mixture was extracted three times with 500 µL of EtOAc, followed by evaporation of the organic phase in vacuo. The residues were dissolved in 600 µL of MeOH and analyzed using n high-performance liquid chromatogra- phy system (Hitachi High-Technologies Co., Tokyo, Japan), based on the method described by Ro et al. (Ro et al. 2001).

Promoter cloning and analysis

The SdCPR and SdC4H promoter sequences (5′ untrans- lated leader regions) were obtained using a Universal GenomeWalker 2.0 Kit (Takara Bio Inc.). The PCR prod- ucts were cloned into a pGEM-T easy vector and then sequenced. PlantCare (http://bioinformatics.psb.ugent.be/ webtools/plantcare/html/) (Lescot et al. 2002) and PLACE (https://www.dna.affrc.go.jp/PLACE/?action=newpl ace) (Higo et al. 1999) were used for identification of cis-elements.

Real-time qPCR

Real-time qPCR was performed using Brilliant III Ultra- Fast SYBR Green QPCR Master Mix (Agilent Technolo- gies, Santa Clara, CA, USA) on an Mx3005p real-time QPCR system (Agilent Technologies). The S. dulcis GAPDH gene (JF718777) was used for normalization. The primer sequences used in the qPCR study are listed in Additional file 1: Table S1. Calibration curves were pro- duced for each of the primer pairs and quantification was performed using the MxPro software (Agilent Technolo- gies). Each sample was tested three times and each mRNA expression value was expressed as mean ± standard devia- tion (SD).

Homology modelling and prediction of 3-D structure of SdCPR

The 3-D structure of SdCPR was constructed using the PHYRE2 server (http://www.sbg.bio.ic.ac.uk/~phyre 2/html/page.cgi?id=index) (Kelley et al. 2015) using the crystal structure of Rattus norvegicus CPR (PDB ID: 1J9Z) as a template. Protein model refinement was performed using KoBaMIN server2012 (http:// chopra-modules.science.purdue.edu/modules/kobam in/html/).

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Structurally, evolutionary, and functionally important regions were identified in deduced protein sequence by ConSurf (https://consurf.tau.ac.il/). Topol- ogy of the modelled SdCPR protein was analyzed using PDBSum (http://www.ebi.ac.uk/thornton-srv/databases/cgi-bin/pdbsum/GetPage.pl?pdbcode=index.html).

Results

Isolation of a full-length cDNA of CPR from S. dulcis

Based on the conserved region of a previously isolated plant CPR, degenerate primers were designed for the P450- and NADPH-binding region, which are highly con- served motifs in the amino acid sequence of higher plant derived CPRs. PCR was performed using cDNA pre- pared from Scoparia leaves as a template. A full-length CPR cDNA was obtained and named SdCPR (Accession number: KF306080). The nucleotide sequence of SdCPR contained an ORF of 2142 bp, and a predicted 713-amino acids protein sequence (estimated molecular weight: 78.5 kDa, PI: 5.09). The SdCPR ORF had conserved bind- ing domains for FMN, FAD, NADPH, and P450, and the membrane anchor was present at the N-terminus (Fig. 1). The SdCPR protein sequence shared 77% sequence iden- tity with pea (Pisum sativum, PsC450R1) and 67% with ashwagandha (Withania somnifera, WsCPR1) as well as 64% and 74% with Arabidopsis (Arabidopsis thaliana, ATR1 and ATR2), and 68% and 77% with cotton (Gos- sypium hirsutum, GhCPR1 and GhCPR2).

CPRs can be classified into class I and class II based on the length of the N-terminal hydrophobic region (Ro et al. 2002). The N-terminal sequences of GhCPR1, WsCPR1, and ATR1 (belonging to class I CPRs) were revealed to be shorter sequence. In contrast, SdCPR con- tained a Ser/Thr rich extended N-terminal region, like other class II CPRs (PsC450R1, GhCPR2, and ATR2) (Fig. 1). Phylogenetic analysis also showed that SdCPR belong to class II group (Fig. 2). In addition, DNA blot- ting analysis showed that a single copy of SdCPR was pre- sent in the S. dulcis genome (Additional file 1: Fig. S2), and the result was identical to our transcriptome analysis (Yamamura et al. 2017).

Heterologous overexpression and catalytic parameters of recombinant SdCPR To examine the catalytic activity of SdCPR, the SdCPR gene was subcloned into pET-28b and used to trans- showed cytochrome c reduction activity in an NADPH- dependent manner (Table

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1); however, this activity was not detected in the absence of NADPH (data not shown). Cytochrome c activity was not observed in the presence of NADH Subsequently, the reduc- tion in activity of the recombinant SdCPR against vari- ous electron acceptors was examined. Cytochrome c, DCPIP, and $K_3Fe(CN)_6$ were all active as electron accep- tors The kinetic parameters K_m and V_{max} of SdCPR for NADPH and cytochrome c are hown in The K_m and V_{max} of SdCPR were 4.6 ± 0.9 μM and 2.3 ± 0.1 μmol min−1 mg protein−1 for NADPH,Effect of wounding and MJ and SA on SdCPR and SdC4H gene expression levels From promotor analysis, we speculated that the expres- sion levels of SdCPR and SdC4H are more likely to be inducible by wounding and elicitors.

Therefore, we further investigated the genes expression patterns of SdCPR and SdC4H in S. dulcis leaves after mechanical wounding and elicitor treatments. SdCPR mRNA levels were increased 1.5-fold within 3 − 6 h after wounding and MJ/SA treatment (Fig. 4b). SdCPR expression was significantly enhanced by wounding, increasing 1.5-fold within 3 h after wounding treatment. SdC4H transcript levels were significantly enhanced within the first 1 h after wounding, 3 h after MJ treatment, and 6 h after SA elicitation (Fig. 4b). There was a time correlation between changes in the expression of both genes. Our results indicated that SdCPR and SdC4H expression levels were induced in response to wounding and elici- tor (MJ and SA), which were consistent with the identi- fied cis-elements.

Prediction of 3-D structure

Based on the structure of Rattus norvegicus CPR (PDB ID: 1J9Z), a predicted 3-D structure of SdCPR was con- structed using a bioinformatics tool. The P450 binding pocket was also presented in the predicted 3-D structure of SdCPR. Subsequently, docking experiments with FMN, FAD, and NADP+ were con- ducted to investigate the positional relationship at the active center. FMN, FAD, and NADP+ molecules were all located in the active pocket, and it was speculated that they play an important role in the reaction of P450. The amino acid residues with high scores (in red) were functional and structural residues of SdCPR .

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Discussion

CPRs are membrane bound proteins localized in the ER, and they function to transfer electrons from NADPH through FAD and FMN to the heme iron center of the various P450 enzymes. It was reported that only one CPR gene is present in yeast, insects, and animals (Porter et al. 1990). The CPR only serves as a redox partner to support various P450 functions in yeast, insects, and animals. On the other hand, filamentous fungi possess one to multiple CPRs, and P450-CPR fusion enzymes have been discovered in some species (Lah et al. 2008). Higher plants also con- tain one to three paralogs of CPRs with different amino acid lengths and regulatory mechanisms (Rana et al. 2013). For instance, two CPRs from Arabidopsis, cot- ton, ashwagandha, and centaury (Centaurium eryth- raea) belong to class I and class II groups, respectively (Mizutani and Ohta 1998; Schwarz et al. 2009; Yang et al. 2010; Rana et al. 2013). Class I CPRs are involved in growth, development, and metabolism; therefore, these CPRs are constitutively expressed in plants (Yang et al. 2010). On the other hand, class II CPRs have been implicated in plant defense systems against envi- ronmental stresses.

In some plant species, only one CPR gene has been reported, such as in coleus (Sole- nostemon scutellarioides), perilla (Perilla frutescens), Catharanthus roseus, Lotus japonicus, pea, and Croton stellatopilosus, which are categorized as class II CPRs (Meijer et al. 1993; Brosché et al. 1999; Eberle et al. 2009; Sintupachee et al. 2015; Fujiwara and Ito 2017). It is assumed that S. dulcis has only one CPR gene from DNA blotting (Additional file 1: Fig. S2) and transcrip- tome analyses (Yamamura et al. 2017). The mRNA of SdCPR was detected in all tissues of S. dulcis plants (Fig. 4a), indicating that the only one SdCPR is widely expressed in S. dulcis to support oxidation reactions involving over 200 P450s in metabolism.

The reductase activity of cytochrome c by recom- binant SdCPR was clearly dependent on NADPH but not on NADH. Similarly, cytochrome c was reduced by CPRs from mung bean (Vigna radiata), poplar (Populus trichocarpa x Populus deltoids), parsley (Petroselinum crispum), and cotton in an NADPH-dependent manner (Shet et al. 1993; Koopmann and Hahlbrock 1997; Ro et al. 2002; Yang et al. 2010). On the other hand,

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house fly (Musca domestica) CPR catalysis of cytochrome c reduc- tion involves NADH as an electron donor (Murataliev et al. 1999). Of note, Döhr et al. reported that the sub- stitution of human CPR Trp-676 with alanine resulted in an enzyme that had about 1000-fold higher specificity for NADH than the wild-type enzyme (Döhr et al. 2001). This data establishes an important role for Trp-676 in NADH binding and recognition, which may provide a functional NADH-dependent P450 monooxygenase system.

Plant P450s play an important role in the biosynthesis of secondary metabolites and are often induced by various stresses. In previous reports, it was demonstrated that the biosynthesis of SDB, a tetracyclic diterpene in S. dulcis, is markedly activated by the MJ and yeast extract treat- ments (Nkembo et al. 2005; Yamamura et al. 2014). It is clear that a large number of P450s are responsible for not only SDB biosynthesis but also in the other biosynthetic reactions of secondary metabolites in S. dulcis (Yama- mura et al. 2017). Among P450s, C4H is a key enzyme in phenylpropanoid biosynthetic pathways such as PAL and 4CL and is known to be inducible by wounding and elicitors (Dixon and Paiva 1995; Bell-Lelong et al. 1997; Mizutani et al. 1997; Akashi et al. 1998). Similarly, Arabidopsis ATR2 expression was induced by wounding and light stress (Mizutani and Ohta 1998), and cotton GhCPR2 expression was induc- ible by wounding and fungal elicitor treatment (Yang et al. 2010). Two CPRs (ATR2 and GhCPR2) belong- ing to class II were induced by stress or elicitors and are likely to be involved in secondary metabolism (Zhao et al. 2018). Based on these reports, we attempted to prove that expression of SdCPR and SdC4H is inducible by stress. The results showed that a variety of elements related to different stress responses such as defense, light, elici- tor treatment, and wounding were observed in both the SdCPR and SdC4H promotor regions (Table 4). In sup- port of this result, both gene transcripts were strongly enhanced in response to different types of stresses such as wounding and MJ and SA treatment (Fig. 4b). There- fore, it is suggested that the SdCPR and SdC4H play an important role in stress-induced defense responses in S.dulcis.

Conclusions

We isolated and characterized a novel NADPH-P450 reductase from S. dulcis,

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which is member of the class II CPRs. SdCPR activities in reducing cytochrome c, DCPIP, and $K_3Fe(CN)_6$, and in supporting P450 monooxygenase (SdC4H) were determined using recombinant proteins produced in E. coli. Expression analysis indicated that both SdCPR and SdC4H transcripts were induced by elic- itor treatment and wounding, which was fully consistent with the identified promoter cis-elements. SdCPR may be helpful to clarify the SDB biosynthetic mechanisms involving multiple P450s in S. dulcis. Our study estab- lished a platform to characterize the P450s involved in plant metabolism.

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