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Characterization of two NADPH: Cytochrome P450 reductases from cotton (*Gossypium hirsutum*)

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ABSTRACT

Cytochrome P450 monooxygenases (P450s) are commonly involved in biosynthesis of endogenous compounds and catabolism of xenobiotics, and their activities rely on a partner enzyme, cytochrome P450 reductase (CPR, E.C.1.6.2.4). Two CPR cDNAs, GhCPR1 and GhCPR2, were isolated from cotton (*Gossypium hirsutum*). They are 71% identical to each other at the amino acid sequence level and belong to the Class I and II of dicotyledonous CPRs, respectively. The recombinant enzymes reduced cytochrome *c*, ferricyanide and dichlorophenolindophenol (DCPIP) in an NADPH-dependent manner, and supported the activity of CYP73A25, a cinnamate 4-hydroxylase of cotton. Both *GhCPR* genes were widely expressed in cotton tissues, with a reduced expression level of *GhCPR2* in the glandless cotton cultivar. Expression of *GhCPR2*, but not *GhCPR1*, was inducible by mechanical wounding and elicitation, indicating that the *GhCPR2* is more related to defense reactions, including biosynthesis of secondary metabolites.

1. Introduction

Cytochrome P450 monooxygenases (P450s) constitute one of the largest superfamilies of enzymes, with more than 2800 sequences being identified in plants (http://drnelson.utmem.edu/CytochromeP450.html), of which 246 are predicted from Arabidopsis (Nelson et al., 2004). They catalyze most of the oxidative reactions, including hydroxylation, epoxidation, dealkylation, dehydration, and carbon–carbon bond cleavage (Schuler and Werck-Reichhart, 2003). Plant P450s are involved in a variety of metabolic pathways, such as biosynthesis of plant hormones and defensive secondary metabolites (e.g., phytoalexins), and in detoxification of exogenous chemicals such as herbicides (Chapple, 1998; Feldmann, 2001; Werck-Reichhart et al., 2000).

The catalytic activities of most eukaryotic P450 monooxygenases rely on cytochrome P450 reductase (CPR, E.C.1.6.2.4) as a partner. CPRs facilitate P450s by transferring two electrons from NADPH to P450s via two prosthetic groups, flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) (Vermilion et al., 1981). Besides working as a partner for P450 monooxygenases, heme oxidation by mammalian heme oxygenase requires CPR and O_2 (Wang and de Montellano, 2003). In yeast, CPR also affects ferrireductase activity, probably by transferring electrons to the flavocytochrome ferric reductase (Lesuisse et al., 1997). CPR is also known to transfer electrons to cytochrome *b5* and form part of an electron transport chain for fatty acid and sterol desaturases on the endoplasmic reticulum (ER) (Fukuchi-Mizutani et al., 1999; Ilan et al., 1981). In plants, it has been shown that CPR activities increased substantially in response to xenobiotics, such as phenobarbital (Reichhart et al., 1980) and salt (Brankova et al., 2007); however, the physiological roles of CPR require further elucidation.

A large number of cytochrome P450 reductases have been isolated and characterized from various plant species, such as *Vigna radiata* (Shet et al., 1993), *Catharanthus roseus* (Meijer et al., 1993), *Arabidopsis thaliana* (Mizutani and Ohta, 1998; Urban et al., 1997), *Petroselinum crispum* (Koopmann and Hahlbrock, 1997) and *Helianthus tuberosus* (Benveniste et al., 1986). Sequences of CPR cDNAs are also available for some medicinal plants, such as *Artemisia annua* (GenBank accession number: EF197890). Although common flavin and NADPH-binding sites are recognizable, plant CPRs share relatively low sequence identities with those from animals and fungi (30–40%), but are highly identical within flowering plants (65–80%). It has been found that most organisms, including animals, yeasts, have only one CPR gene in each genome, whose expression product interacts with different P450s (Porter et al., 1990; Simmons et al., 1985). Fungal genomes may have one (*Gibberella fujikuroi*) or multiple (*Rhizopus nigricans*) CPR genes, and in some species, the P450-CPR fusion enzymes are also found (Kunic et al., 2001; Lah et al., 2008; Malonek et al., 2004). Higher plants usually have dual (Benveniste et al., 1991; Koopmann and Hahlbrock, 1997) or multiple (Ro et al., 2002) CPRs with different

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relative molecular weights, subcellular localizations and regulatory mechanisms have been reported. In some plant species, although only one CPR gene was reported (Meijer et al., 1993; Rosco et al., 1997), it did not exclude the possibility that multiple CPR genes exist in their genomes. Different members of the CPR gene family in a plant are usually differentially regulated. For example, the *A. thaliana* genome has two CPR genes, *ATR1* and *ATR2*; while *ATR1* is constitutively expressed, *ATR2* is inducible by environmental stimuli, such as wounding and light treatments (Mizutani and Ohta, 1998). In parsley (*P. crispum*), however, only one of the two CPRs isolated is responsive to pathogen infection (Koopmann and Hahlbrock, 1997). It was thus proposed that plants deploy distinct CPR isoforms to cope with the high reductive demand for the P450-mediated reactions in stressed conditions (Ro et al., 2002).

Cotton is one of the most important crops and cotton plants accumulate a large amount of gossypol and related sesquiterpene aldehydes. These secondary metabolites function as phytoalexins and protect the plants against pathogens and herbivores (Essenberg et al., 1990; Mao et al., 2007). Biosynthesis of gossypol starts from (+)-δ-cadinene synthase, a sesquiterpene synthase, and requires the participation of P450 monooxygenases as several hydroxyl groups exist in their structures. Previously, we isolated and identified one of these P450s, CYP706B1, which catalyzes 8-hydroxylation of (+)-δ-cadinene, a first hydroxylation step of gossypol biosynthesis (Luo et al., 2001b). A recent report showed that CPR activities in cotton leaves were increased after salt (sodium chloride) treatment (Brankova et al., 2007). Here, we report two isoforms of P450 reductase from *Gossypium hirsutum*, GhCPR1 and GhCPR2. While *GhCPR1* was constitutively expressed in plants, *GhCPR2* expression was inducible by either wounding or elicitor treatments.

2. Results

2.1. Isolation and sequence analysis of GhCPR1 and GhCPR2

By searching the *G. hirsutum* EST database (www.ncbi.nlm.nih.gov) with two divergent Arabidopsis CPR cDNA sequences, *ATR1* (NM_118585) and *ATR2* (NM_119167) (Mizutani and Ohta, 1998), two ESTs (ES819464 and DT466618) with 74% nucleotide sequence identities were retrieved, and their 5′ and 3′ flanking regions were obtained by RACE (rapid amplification of cDNA ends). The full-length cDNAs were then isolated by PCR and designated GhCPR1 and GhCPR2, respectively (GenBank accession numbers: FJ719368 and FJ719369).

The GhCPR1 cDNA contains an open reading frame (ORF) encoding a protein of 694 amino acids, with a predicted relative molecular weight of 77.14 kD. GhCPR2 encodes a 711-amino acid protein of 78.59 kD. By aligning deduced sequences of GhCPR1

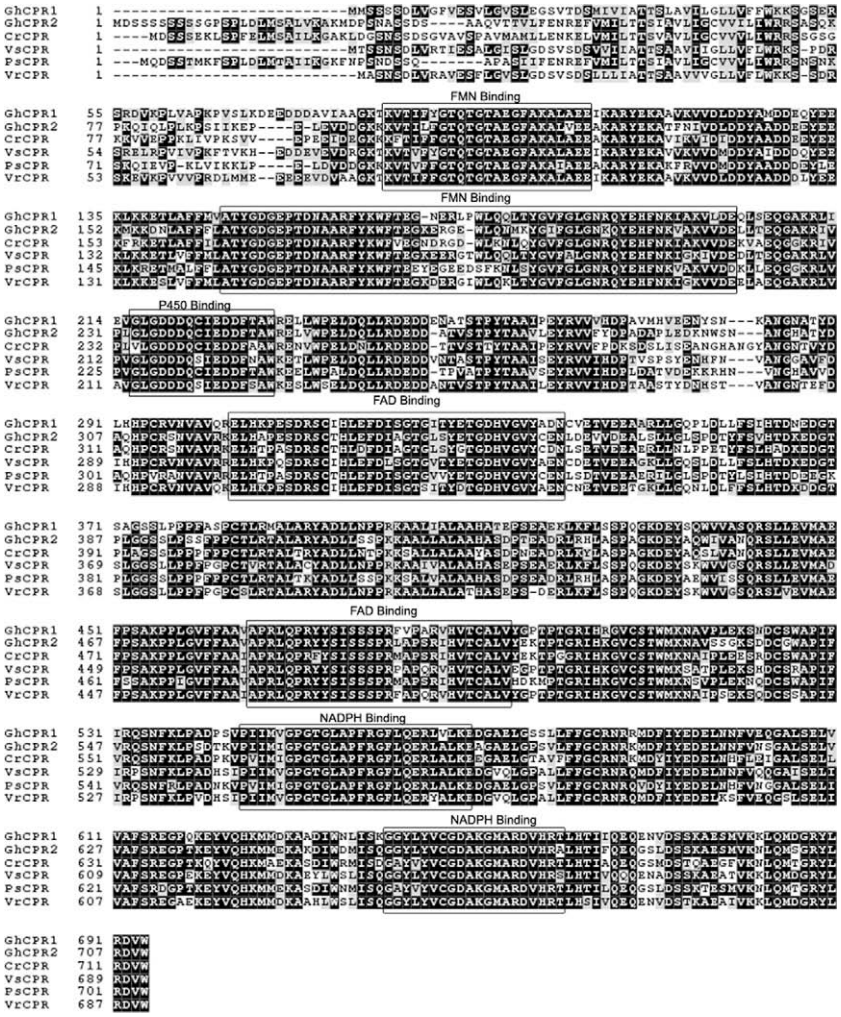

Fig. 1. Alignment of amino acid sequences P450 reductases (CPRs) of dicot plants. The deduced amino acid sequences of GhCPR1 (FJ719368) and GhCPR2 (FJ719369) were aligned with those of CPRs from *C. roseus* (CrCPR, X69791), *Pisum sativum* (PsCPR, AF002698), *V. radiata* (VrCPR, A47298) and *Vicia sativa* (VsCPR, Z26252) by the program Clustal W. The putative functional regions involved in the interaction with FMN, FAD, NADPH and P450 are indicated.

and GhCPR2 with other CPRs from taxonomically diverse species, all functional domains involved in the binding of the P450 monooxygenase, and the cofactors of FMN, FAD and NADPH, were identified (Fig. 1).

Amino acid sequence comparison showed that these two cotton CPRs are 71% identical to each other, and both share high sequence identities with CPRs from other flowering plant species (65–80%). In general, the N-terminal membrane anchoring region shows the highest divergence, whereas the rest of the protein is conserved among plant CPRs (Fig. 1).

A previous sequence analysis suggested that CPRs from dicots can be divided into two classes, and each species has two or more CPRs that belong to either class, respectively. Class I contained such members as Arabidopsis ATR1 and hybrid poplar (*Populus trichocarpa* × *Populus deltoids*) CPR1, and Class II was exemplified by Arabidopsis ATR2 and hybrid poplar CPR2/3 (Ro et al., 2002). Phylogenetic analysis of CPR proteins from cotton and other plant species using MEGA 4 program and neighbor-joining algorithm (Kumar et al., 2008) indicated that GhCPR1 was grouped with ATR1 of Class I, and GhCPR2 with ATR2 of Class II, respectively (Fig. 2).

2.2. Catalytic parameters of recombinant GhCPRs

To characterize the activities of GhCPR1 and GhCPR2, both enzymes were produced in *Escherichia coli* as recombinant proteins. After solubilization from membrane and affinity purification, the purity of the CPR proteins was verified via SDS–polyacrylamide gel electrophoresis (SDS–PAGE).

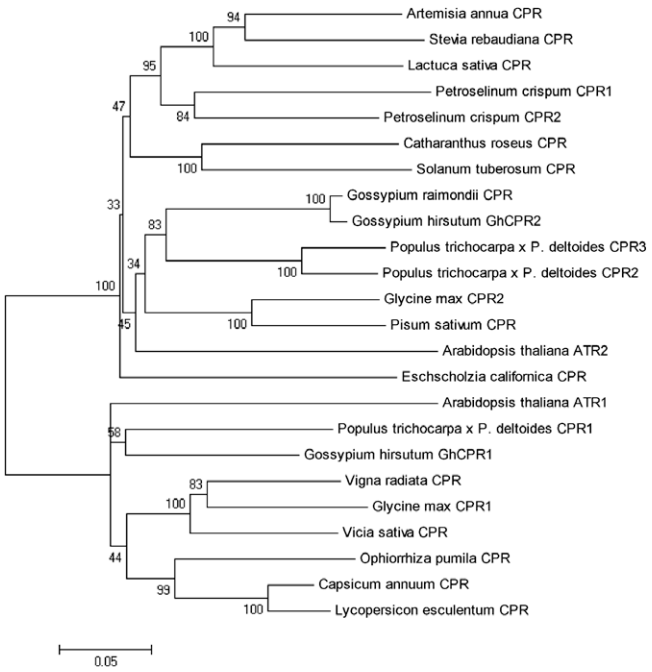

Fig. 2. Phylogenetic analysis of characterized and putative dicot CPRs based on amino acid sequences. Tree reconstruction was performed with the neighbor-joining algorithm using the MEGA (Version 4) program. Bootstrap values (percent of 1000 replicates) for each cluster is shown at the nodes. CPR sequences of the following plants were used for analysis: *A. annua* (EF197890); *Stevia rebaudiana* (DQ269454); *P. crispum* (CPR1: AF024635, CPR2: AF024634); *A. thaliana* (*ATR1*: X66016, *ATR2*: X66017); hybrid poplar (*P. trichocarpa* × *P. deltoids*, CPR1: AF302496, CPR2: AF302497, CPR3: AF302498); *P. sativum* (AF002698); *C. roseus* (X69791); *Eschscholzia californica* (ECU67186); *V. radiata* (A47298); *V. sativa* (Z26252); *Ophiorrhiza pumila* CPR (AB086169); *Capsicum annuum* (EU616557); *Glycine max* (CPR1: AY170374; CPR2: AK285782); *Lycopersicon esculentum* (BT013756) and *G. hirsutum* (GhCPR1: FJ719368, GhCPR2: FJ719369). CPRs of following species were assembled from EST database: *Solanum tuberosum*, *Lactuca sativa*, and *Gossypium raimondii*.

The content of flavins of both CPR proteins was measured. The total flavin contents of GhCPR1 and GhCPR2 were 24 and 22.5 nmole/mg protein, respectively. One mole GhCPR1 contained 0.87 mol of FAD and 0.83 mol of FMN, whereas GhCPR2 contained 0.91 mol of FAD and 0.86 mol of FMN. When the cytochrome *c* reducing assay was performed in the presence of 5 μM FMN, GhCPR1 and GhCPR2 showed only 6% and 5% increase in activity, respectively. This suggested that the preparation were only slightly FMN-depleted.

The recombinant GhCPR1 protein showed similar absolute absorption spectrum as CPR proteins from other plant species (Benveniste et al., 1986; Urban et al., 1997), with prominent peaks

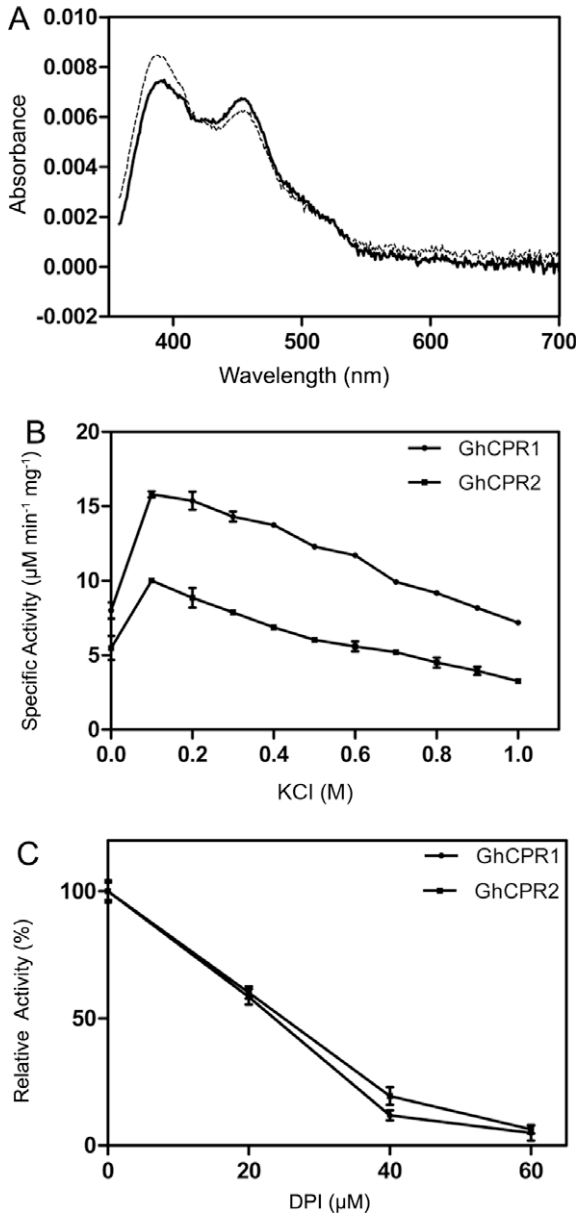

Fig. 3. Spectra and catalytic activities of recombinant GhCPR1 and GhCPR2 proteins. (A) Absorption spectra of the purified recombinant GhCPR1 protein. The one-electron reduced semiquinone form of the protein was prepared by adding 1 mM NADPH to a final concentration of 5 μM, and the spectra were recorded after incubating for 15 min at 25 °C. Solid line: oxidized form; dashed line: semiquinone form. (B) Effects of ion strength on NADPH-cytochrome *c* reducing activities of GhCPR1 and GhCPR2. Activities were measured with various concentrations of KCl. (C) Effects of DPI on GhCPR1 and GhCPR2 activities of reducing cytochrome *c*. The relative activities were normalized to the specific activity of GhCPR1 and GhCPR2 without DPI, respectively.

Table 1
Specific activities of GhCPR1 and GhCPR2 in reducing cytochrome *c* (20 μM), in the presence of 20 μM of NADPH or NADH. Values are presented as mean ± SE. ND, not detected.

	Specific activity (μmol/min/mg protein)	
	NADPH	NADH
GhCPR1	4.78 ± 0.08	ND
GhCPR2	3.30 ± 0.06	ND

Table 2
Reduction of cytochrome *c*, DCPIP and $K_3Fe(CN)_6$, all at 20 μM, by recombinant GhCPR1 and GhCPR2. Values are presented as mean ± SE.

	Specific activity (μM/min/mg protein)		
	Cytochrome *c*	DCPIP	$K_3Fe(CN)_6$
GhCPR1	4.69 ± 0.12	9.32 ± 0.22	3.47 ± 0.10
GhCPR2	3.17 ± 0.06	9.84 ± 0.26	2.15 ± 0.05

Table 3
Steady-state kinetic constants of recombinant GhCPR1 and GhCPR2 at 25 °C, pH 7.4. Determination of kinetic parameters for cytochrome *c* was performed in a reaction mixture containing 100 μM NADPH and various amounts of cytochrome *c*, and kinetic parameters for NADPH was determined by using 100 μM cytochrome *c* as substrate and various amounts of NADPH. Values were obtained by Lineweaver–Burke plot analysis, and are presented as mean ± SE.

	NADPH		Cytochrome *c*	
	GhCPR1	GhCPR2	GhCPR1	GhCPR2
V_{max}(μmol/min/mg^{-1})	3.0 ± 0.1	7.8 ± 0.4	3.1 ± 0.1	7.7 ± 0.3
K_m (μM)	4.6 ± 0.2	5.6 ± 0.6	1.2 ± 0.1	1.6 ± 0.2
k_{cat} (min^{-1})	231.4	613.0	239.2	605.2
k_{cat}/K_m	50.3	109.5	199.3	378.3

at about 450 and 380 nm, respectively, typical of a flavoprotein. Aerobic treatment of the reductase with 10 μM NADPH produced a spectrum having a broad peak of approximately 600 nm, which

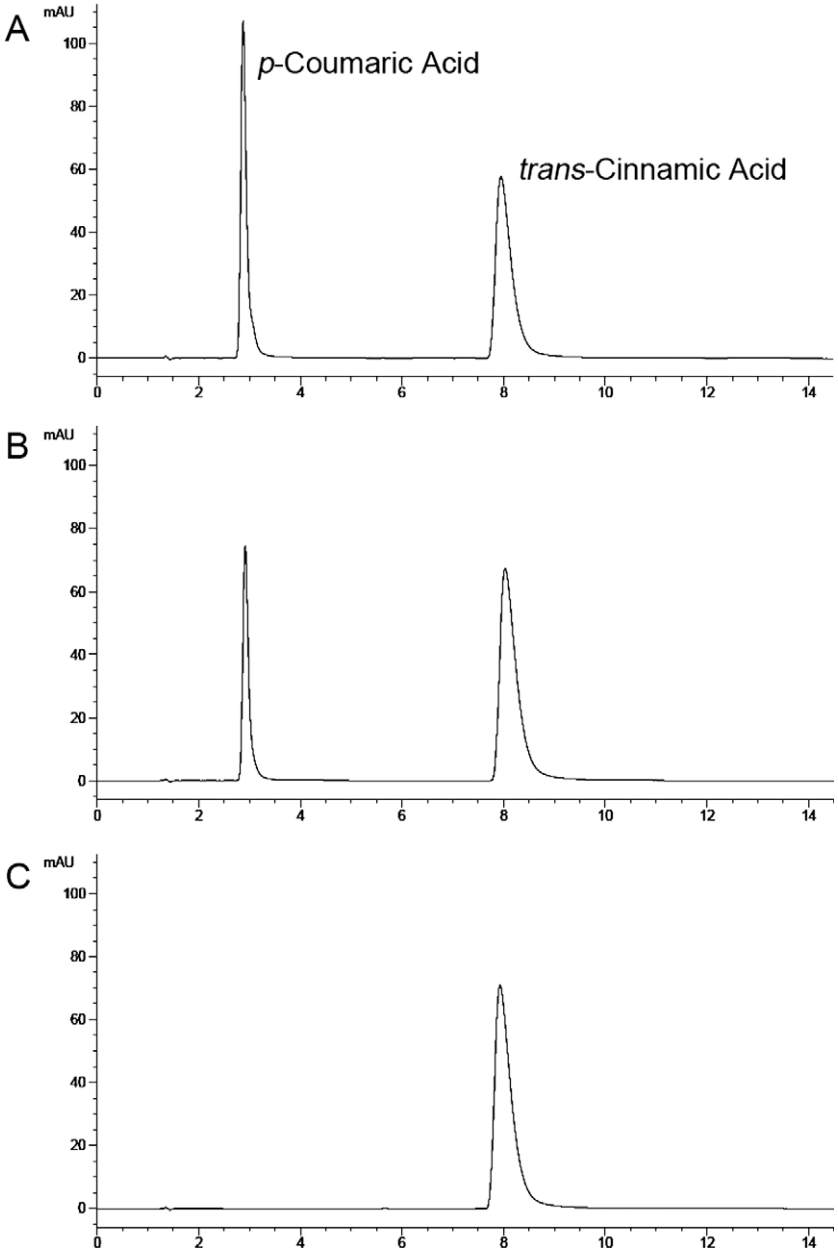

Fig. 4. HPLC separation of the reaction mixture of recombinant GhCPR1- and GhCPR2-coupled CYP73A25 activity, showing formation of *p*-coumaric acid. The *E. coli* cell lysates containing recombinant GhCPR1 and CYP73A25 (A), or GhCPR2 and CYP73A25 (B), or CPY73A25 along (C), were used for cinnamate 4-hydroxylase assay in 50 mM phosphate buffer, pH 7.4.

is typical for the semiquinone form of flavoproteins (Fig. 3A). The purified GhCPR2 protein showed similar spectrum as GhCPR1 (data not shown).

A previous report showed that phosphate buffer interfered with the binding of NADPH and cytochrome *c*, increasing K_m for the substrates 3–5-fold (Murataliev and Feyereisen, 2000). To avoid the inhibitory effect, most of the assays were carried out in 50 mM Tris–HCl buffer at pH 7.4, unless otherwise indicated. The recombinant GhCPR1 and GhCPR2 were assayed for NADH- or NADPH-dependent cytochrome *c* reduction activities, respectively (Table 1). Strong cytochrome *c* reductase activities were observed for both GhCPRs when NADPH was added. By contrast, NADH did not support either GhCPR1 or GhCPR2 for the reducing reactions, indicating that both GhCPRs utilize NADPH specifically as the electron donor.

Compared to the electron donor (NADPH), the requirement of CPRs for electron acceptor is relatively less specific: cytochrome *c*, $K_3Fe(CN)_6$, and dichlorophenolindophenol (DCPIP) can all serve as acceptors (Table 2). The kinetic characteristics of GhCPR1 and GhCPR2 for reducing cytochrome *c* were determined, respectively, at pH 7.4. In general, GhCPR1 had higher affinities for the substrates as revealed by K_m value, whereas GhCPR2 showed higher activities (higher k_{cat} value) than GhCPR1. As a result, the catalytic efficiency (k_{cat}/K_m value) of GhCPR2 was higher than that of GhCPR1 (Table 3).

Effect of ionic strength on CPR proteins was investigated. As shown in Fig. 3B, cytochrome *c* reducing activities of both GhCPR1 and GhCPR2 increased to about twofold in the presence of 0.1 M KCl, then decreased at higher concentrations of KCl. In 1 M KCl, the specific activity was similar to (GhCPR1) or slightly lower than (GhCPR2) that without KCl. Similar results were observed with NaCl (data not shown).

Diphenyleneiodonium (DPI) is a widely used non-competitive inhibitor of flavoenzymes, particularly those NADPH-dependent, by binding to the catalytic center (O'Donnell et al., 1994). The cytochrome *c* reduction activities of GhCPR1 and GhCPR2 were inhibited to about 60% by 20 μM DPI, and the inhibition was more than 90% when DPI concentration was increased to 60 μM (Fig. 3C). These data are consistent with the results obtained from *Trypanosoma cruzi* CPRs (Portal et al., 2008).

2.3. GhCPRs supported P450 monooxygenase activity

To demonstrate the function of GhCPRs as cytochrome P450 reductase, a cotton cytochrome P450, CYP73A25 (Luo et al., 2001a), which shares 95% protein sequence identity with

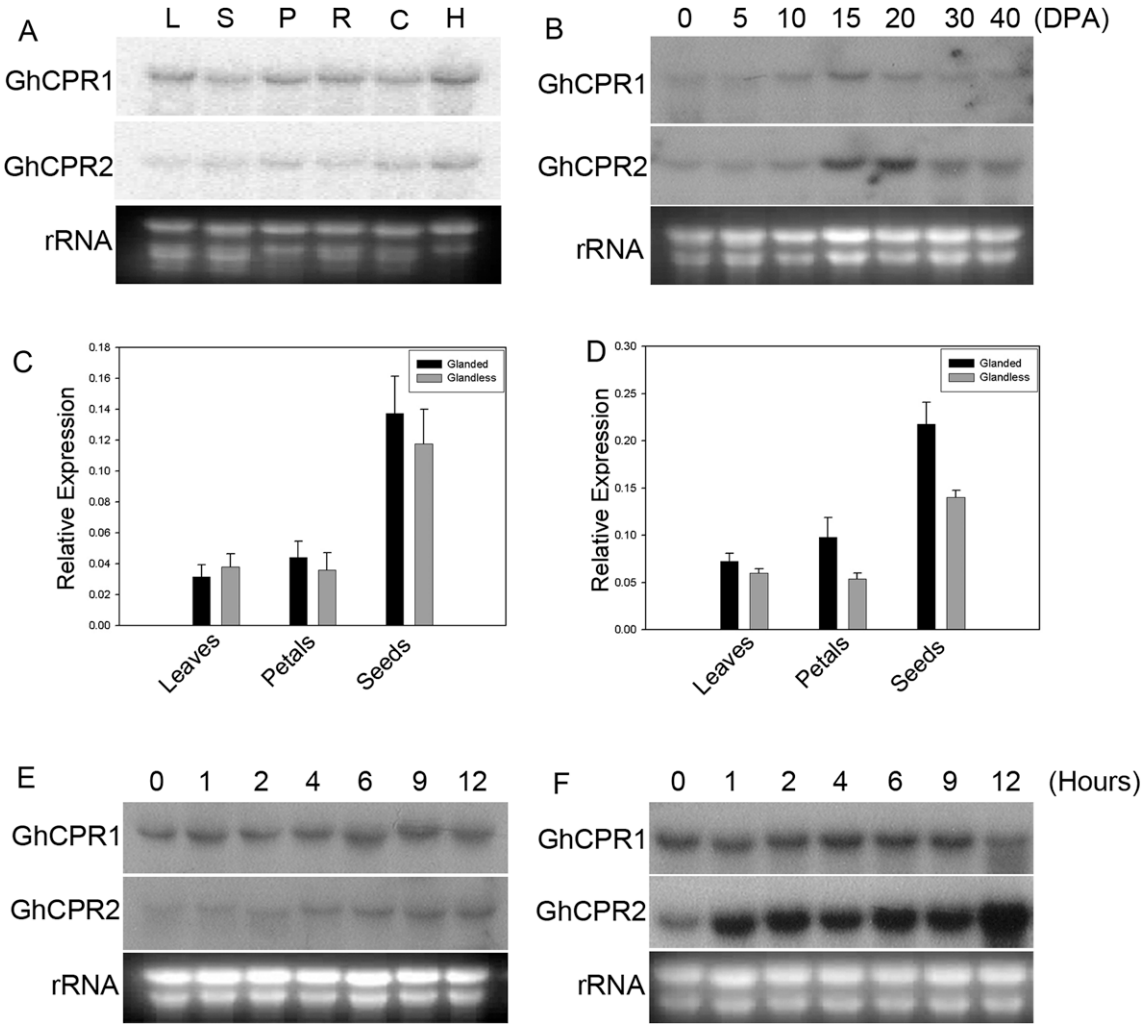

Fig. 5. Expression patterns of GhCPR1 and GhCPR2 in *G. hirsutum* L. (A) Expression of GhCPR1 and GhCPR2 in cotton flowering stage plants and seedlings (7-day-old). L: leaf; S, sepal; P, petal; R, root; C, cotyledon; H, hypocotyl. (B) Expression of GhCPR1 and GhCPR2 in developing seeds (ovules) collected at 0, 5, 10, 15, 20, 30 and 40 DPA. (C) and (D) Real-time PCR analysis of GhCPR1 (C) and GhCPR2 (D) expression in leaf, petal (0 DPA) and seed (ovule, 15-DPA) of glanded and glandless cotton cultivars, respectively. (E) Effect of wounding on GhCPR1 and GhCPR2 expression levels in hypocotyl. Hypocotyls of 7-day-old cotton seedlings were cut into 1-cm-long fragments and incubated in a Petri dish containing Murashige and Skoog medium. (F) Effect of *Verticillium dahliae* elicitor on GhCPR1 and GhCPR2 gene expression in cotton suspension cultured cell.

Arabidopsis cinnamate 4-hydroxylase CYP73A5, was used in the assay. The ORFs of CYP73A25 and GhCPR1 or GhCPR2 were cloned in the bacterial coexpression vector pETDUET-1 and expressed in *E. coli*. After induction, the cell lysate was incubated with cinnamic acid. Analysis by high performance liquid chromatography (HPLC) showed that the expected product *p*-coumaric acid, was formed. By contrast, cells expressing only CYP73A25 protein without either CPR did not catalyze cinnamic acid hydroxylation (Fig. 4). Both CPRs of cotton are therefore active in facilitating P450 monooxygenase.

2.4. Expression patterns of GhCPR1 and GhCPR2 in G. hirsutum

The expression of *GhCPR1* and *GhCPR2* in cotton plant was investigated by Northern blot (Fig. 5A), which showed that transcripts of *GhCPR1* were present in all tissues examined, including leaf, sepal, petal, cotyledon, hypocotyl and root. *GhCPR2* also was widely expressed, but its steady state transcript level was much lower than that of *GhCPR1*.

During cotton seed development, both *GhCPR1* and *GhCPR2* were expressed in the embryo (Fig. 5B), where cotyledons took a major part. *GhCPR1* was expressed at a relatively constant level throughout the developmental stages investigated (0–40 days post anthesis, DPA), with a slight up-regulation at 15 DPA, possibly due to the uneven loading of RNA sample, whereas *GhCPR2* showed a dramatic increase of transcript abundance around 15–20 DPA, in parallel with a highly active biosynthesis of gossypol as previously reported (Tan et al., 2000).

Cotton plants accumulate a high amount of secondary metabolites, such as gossypol, which are stored in glands of aerial tissues and in the epidermis and subepidermis of roots (Liang et al., 2000). Compared to the widely cultivated glanded cottons, a few of the cultivars are glandless and therefore do not accumulate gossypol in aerial tissues. The gossypol biosynthesis pathway genes, including those encoding *(+)-δ-cadinene synthase* and *(+)-δ-cadinene-8-hydroxylase (CYP706B1)*, showed an obvious down-regulation in the glandless cultivar (Luo et al., 2001b; Tan et al., 2000). To investigate the relationship between *GhCPRs* and biosynthesis of gossypol and related sesquiterpene phytoalexins, the expression of both *CPR* genes in glanded and glandless cotton cultivars was analyzed by real-time PCR. In leaves, petals and the 15-DPA developing ovules (seeds), expression of *GhCPR1* did not change markedly between the glanded and glandless cultivars. However, the *GhCPR2* expression level was about 40% lower in the glandless petal and ovule (Fig. 5C and D), suggesting that of the two CPRs, *GhCPR2* is more involved in gossypol biosynthesis.

Increased biosynthesis of sesquiterpene aldehydes in cotton tissues and cultured suspension cells can be induced by fungal and bacterial elicitors and by mechanical wounding, and expression of all the enzyme genes characterized so far, including the P450 monooxygenase gene *CYP706B1*, was induced during elicitation (Liu et al., 1999; Luo et al., 2001b). Therefore, enhanced participation of P450 reductase was anticipated. We then investigated if expression of the two cotton CPR genes was inducible. Northern blots showed that in hypocotyl *GhCPR1* transcript level did not change after mechanical wounding (Fig. 5E), and the expression in suspension cultured cells was largely unchanged after treatment with the elicitor prepared from *Verticillium dahliae* (Vde) (Fig. 5F), which was shown to induce gossypol biosynthesis and expression of related genes (Chen et al., 1995; Luo et al., 2001b). On the contrary, although *GhCPR2* had a lower expression level than *GhCPR1* in hypocotyl, its transcript abundance was clearly increased after mechanical wounding (Fig. 5E). In suspension cells, *GhCPR2* transcription was drastically induced by elicitation to a level that was much higher than that of *GhCPR1* (Fig. 5F), suggesting that *GhCPR2* became the major CPR in elicitor-treated cells. Taken together, these data demonstrate that while *GhCPR1* is a constitutively expressed gene, *GhCPR2* responses to exogenous stimuli.

3. Discussion

In this study, two NADPH:cytochrome P450 reductases from *G. hirsutum*, GhCPR1 and GhCPR2, were isolated and investigated for their catalytic properties and gene expression patterns. Sequence comparison confirmed previous finding that CPRs of dicotyledon plants can be classified into two clusters (Ro et al., 2002). GhCPR1 and GhCPR2 belong to Class I and Class II, respectively. The dicot plant species, such as *Glycine max*, hybrid poplar, *A. thaliana* and *G. hirsutum* investigated herein, have divergent CPRs in both classes. The sequence similarities within each class, although from different taxa, are normally higher than those between different class CPRs from a single plant species.

Both GhCPR1 and GhCPR2 have a relative spectrum of electron receptors, including cytochrome *c*, DCPIP, ferrocyanide and the P450 monooxygenases. However, both GhCPR proteins used NADPH only as electron donor to support the reducing activity. The same electron donor preference was reported for CPRs from other plant species, such as mung bean (Shet et al., 1993), poplar (Ro et al., 2002) and parsley (Koopmann and Hahlbrock, 1997). It was also reported that the CPR of *T. cruzi* could not utilize NADH to support cytochrome *c* reducing activity (Portal et al., 2008). However, CPRs from some insects, such as house fly, can utilize NADH to support cytochrome *c* reducing activity, with nearly four orders of magnitude higher K_m value than that for NADPH, and the maximal rate was about 13% of the rate with NADPH (Murataliev et al., 1999). Interestingly, the human CPR protein with a single amino acid change could utilize NADH as efficiently as NADPH (Dohr et al., 2001), which is an important step towards elucidation of how this specificity was determined.

The P450 reductase activity from rat could be affected by ionic strength. It showed an activity increase as the salt concentrations raised until 0.5 M salts, then kept at a high level of about 2.5-fold than the control (Benveniste et al., 1986). Cotton CPR protein activities were also affected by ionic strength. However, unlike rat CPR, both cotton CPR protein activities were elevated drastically by relatively low salt concentrations, and this activity was inhibited by higher ionic strength (>0.1 M). Similar property was observed with *H. tuberosus* CPR, although the activity was less stimulated (20% increase) at low ionic strength (Benveniste et al., 1986).

Plant P450s play important roles in secondary metabolism, which is often stimulated under stress conditions. In cotton, it is *GhCPR2*, but not *GhCPR1*, that showed a rapid induction of transcription after wounding and elicitor treatments. Similarly, in Arabidopsis, expression the Class II CPR gene *ATR2* was induced in response to the wounding and light treatments, whereas *ATR1* of Class I was constitutively expressed (Mizutani and Ohta, 1998). Two CPR cDNAs of *P. crispum* were isolated and characterized; treatments of parsley cultured cells with fungal elicitor or UV-irradiation induced one of the two CPR genes (Koopmann and Hahlbrock, 1997). Taken together, these data suggest that in dicot plants, in addition to constitutively expressed CPRs, at least one CPR is inducible in providing additional reductase activities under biotic or abiotic stress situations. Interestingly, all the inducible CPRs reported so far are of Class II.

Differential regulation of isozyme genes was also observed for *farnesyl diphosphate synthase* (*FPS*), which provides substrate for sesquiterpene and steroid biosynthesis. Our previous work showed that there are two *FPS* in cotton (*Gossypium arboreum*), and one of them is inducible by fungal elicitation (Liu et al., 1999). Similar phenomenon was observed for *3-hydroxy-3-methylglutaryl-CoA reductase* (*HMGR*), a critical enzyme of mevalonic-acid pathway

(MVA pathway) (Friesen and Rodwell, 2004). In potato, of the three *HMGR* genes, one did not respond to fungal pathogen elicitor, while the other two can be induced by this treatment (Choi et al., 1992). In rice, of the three *HMGRs* one showed a low and inducible expression pattern, whereas the other two showed relatively constitutive expression (Ha et al., 2001; Kim et al., 2004; Nelson et al., 1994). Thus the existence of two or more differentially regulated genes coding for the isoenzyme may grant plants the capability to fine-tune their metabolic flux in response to different physiological conditions. This also suggests that *CPRs* may be part of the regulatory machinery of plant defense reactions, particularly the secondary metabolism.

Cotton provides the most important natural fiber for textiles. In addition, cottonseed is also rich in protein and oil (Sunilkumar et al., 2006). However, the wide distribution of gossypol and other sesquiterpene aldehydes in cotton plants have limited the utilization of cottonseed as a high-quality protein source (Townsend and Llewellyn, 2007). Biosynthesis of these phenolic sesquiterpene aldehydes requires the participation of P450 monooxygenase (Luo et al., 2001b). Characterization of cotton P450 reductases should help further elucidation of biosynthetic pathways of gossypol and other phytoalexins.

4. Concluding remarks

In this study, we isolated and characterized two P450 reductases from *G. hirsutum*, which are members of dicot Class I and Class II CPRs, respectively. Their activities in reducing cytochrome *c*, $K_3Fe(CN)_6$ and DCPIP, and in supporting P450 monooxygenase were determined by using recombinant proteins produced in bacteria. Expression analysis indicated that while *GhCPR1* was expressed constitutively, *GhCPR2* could be induced by mechanical wounding and elicitation. These data suggest that *GhCPR2* plays a major role in defense reactions, including gossypol biosynthesis. These two cotton CPRs will facilitate our understanding of secondary metabolism in cotton.

5. Experimental

5.1. Plant material

Plants of upland cotton (*G. hirsutum* L.), glanded (cv. CCRI12) and glandless (*G. hirsutum* cv. CCRI20) cultivars, were grown in greenhouse at 30 °C. Plant materials, including root, hypocotyl, cotyledon, leaf, flower and seed of 10-, 20- and 30-day post anthesis (DPA), were sampled and frozen immediately in liquid nitrogen, and stored at −80 °C.

Suspension culture of cotton cells and treatment of the cells with the fungal (*Verticillium dahliae*) elicitor were carried out as previously described (Liu et al., 1999). For mechanical wounding treatment, hypocotyls of 7-day-old cotton seedlings were cut into 1-cm fragments and incubated in a Petri dish containing Murashige and Skoog medium for 1, 2, 4, 6, 9, 12 h, respectively. Samples were collected and frozen immediately in liquid nitrogen, and stored at −80 °C.

5.2. Total RNA isolation and analysis

Samples of young leaves or other tissues were ground into fine powder and extracted with extraction buffer containing 0.2 M Tris, pH 8.0, 50 mM EDTA, 1 M NaCl, 1% cetyltrimethyl ammonium bromide (CTAB), and 1% β-mercaptoethanol. The mixture was incubated at 65 °C for 30 min and further extracted twice with 1 volume of phenol/$CHCl_3$ (1:1) and then the supernatant was precipitated by adding 8 M LiCl to a final concentration of 2 M and stored at −20 °C for 2 h, followed by centrifugation at 13,000*g* for 20 min at 4 °C. The RNA pellets were washed with 70% ethanol. After a short drying at room temperature, the pellets were resuspended in diethylpyrocarbonate (DEPC)-treated water. Total RNA obtained was used as template for cDNA synthesis or Northern blot.

For Northern blots, total RNA (10 μg each lane) was resolved on 1.2% (w/v) formaldehyde agarose gel and transferred onto Hybond N+ membrane (GE Healthcare-Amersham, NJ, USA). Probe hybridization was performed overnight at 65 °C in 250 mM sodium phosphate, pH 7.4, containing 1% (w/v) BSA, 7% (w/v) SDS and 1 mM EDTA. Membranes were washed twice for 20 min at 65 °C in 2X SSC with 0.1% (w/v) SDS, followed by one wash at 65 °C in 0.1X SSC and 0.1% (w/v) SDS for 20 min.

For real-time PCR analysis, total RNA was treated with DNase I (Promega, USA), and then reverse transcribed using SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Real-time PCR was performed using the SYBR PrimeScript RT-PCR kit (Takara, Dalian, China) on a Rotor-Gene thermocycler (Corbett Research, Mortlake, NSW, Australia). Cotton *histone 3* gene (AF024716) was used for normalization. Primers used were as follows: his3-F: 5′-GAAGCCTCATCGATACCGTC-3′ and his3-R: 5′-CTACCACTACCATCATGG-3′ for *histone 3*; CPR1RT-F: 5′-GCTCCTCGTTTACAGCCTCG-3′ and CPR1RT-R: 5′-GGACCTTCTCGTGAAAATGC-3′ for GhCPR1; CPR2RT-F: 5′-CCTTCAGCCAAGCCTCCACT-3′ and CPR2RT-R: 5′-TCCTTGGTAGGTCCCTCACG-3′ for GhCPR2. Calibration curves were produced for each of the primer pairs and quantification was performed using the Rotor Gene Analysis Software 6.0 (Corbett Research).

5.3. 5′ and 3′ RACE and amplification of full-length cDNAs

Rapid amplification of cDNA ends (RACE) was performed with the adaptor (QT: 5′-CCAGTGAGCAGAGTGACGAGGACTCGAGCTCAAGCTTTTTTTTTTTTTTTT-3′) and adaptor primers (Q1: 5′-CCAGTGAGCAGAGTGACG-3′; Q2: 5′-GAGGACTCGAGCTCAAGC-3′), as described (Scotto-Lavino et al., 2006). For 5′ RACE, the first strand cDNA was synthesized with gene specific primer CPR1-RT (5′-ACGGTTACCCAGACCAAATA-3′) for GhCPR1, and CPR2-RT (5′-GAACCTGGCTGCATTATCTG-3′) for GhCPR2. The 5′ fragments were amplified with gene specific primers CPR11 (5′-GCCTTTCATTTCCCTCAGTA-3′) and CPR12 (5′-CTAGCAGCGTTATCGGTTGG-3′) for GhCPR1, and CPR21 (5′-GCTCACCGTCTCCATAAGTG-3′) and CPR22 (5′-GCTCACCGTCTCCATAAGTG-3′) for GhCPR2. The 3′ RACE was performed with gene specific primers CPR13 (5′-AGATTGTTGGGTCAACCTCT-3′) and CPR14 (5′-CACACTGACAATGAGGACGG-3′) for GhCPR1, and CPR23 (5′-TTTATGAGAAAACGCCAACA-3′) and CPR24 (5′-CTTGGATGAAGAATGCTGTG-3′) for GhCPR2.

Based on sequencing results of the 3′ and 5′ RACE products, the ORFs of both GhCPRs were amplified in a 50-μL standard pfu PCR system (Stratagene, La Jolla, CA, USA) with following cycling parameters: 94 °C for 3 min, followed by 30 cycles of amplification (94 °C for 30 s, 55 °C for 30 s, 72 °C for 3 min), and an incubation at 72 °C for 10 min. Gene specific primers were CPR1F (5′-ATGAGTTCGAGTTCCGATTT-3′) and CPR1R (5′-TCACCACACATCTCTAAGGTAT-3′) for GhCPR1, and CPR2F (5′-ATGGATTCTTCATCATCATCATC-3′) and CPR2R (5′-TCACCATACATCGCGTAGGT-3′) for GhCPR2.

5.4. Prokaryotic expression of recombinant proteins

The ORFs of GhCPRs were amplified by Pfu DNA polymerase using primers CPR1ORF-F-*Bam*HI (5′-CGGATCCATGAGTTCGAGTTCCGATTTGG-3′) and CPR1ORF-R-*Sal*I (5′-CGTCGACTTATACCCATCACCACACGTCTC-3′) for GhCPR1, and CPR2ORF-F-*Bam*HI (5′-CGGATCCATGGATTCTTCATCATCATC-3′) and CPR2ORF-R-*Eco*RI (5′-CGAATTCTCACCATACATCGCGTAGGT-3′) for GhCPR2. The PCR

products were digested with *Bam*HI and *Sal*I, or *Bam*HI and *Eco*RI, for GhCPR1 and GhCPR2, respectively, and then inserted into the expression vector pET32a, which was used to transform *Escherichia coli* BL21 (DE3).

The *E. coli* BL21 (DE3) cells harboring the expression vector were grown overnight in Luria–Bertani medium with 100 μg mL^{-1} ampicillin at 37 °C in a shaking incubator, then diluted 1:100 into Terrific Broth medium supplemented with 100 μg mL^{-1} ampicillin. Cells were grown at 37 °C at 200 rpm to an A_{600} of 0.5, and then 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was added. The culture was shaking at 200 rpm at 28 °C for 12 h for protein expression. The bacterial cells were collected by centrifugation at 4000 rpm for 5 min at 4 °C, washed twice with 4 °C MOPS buffer (100 mM MOPS, 10% glycerol, 0.2 mM DTT, 1 mM EDTA, adjusted to pH 7.3 with NaOH), and resuspended in the same buffer containing 10 μg mL^{-1} lysozyme (Sigma–Aldrich, USA), kept at 4 °C for 30 min. The recombinant protein purification was carried out as described with modifications (Hull and Celenza, 2000). After sonication on ice, the membranes were collected by centrifugation at 14,000*g* for 30 min at 4 °C and the membrane proteins solubilized in 4 °C MOPS buffer containing 1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) by rocking gently on ice overnight. After solubilization the sample was centrifuged as before and the supernatant containing solubilized membrane proteins was applied to Ni–NTA resins for affinity purification, according to manufacturer's manual (Novagen, Madison, USA).

For the bacterial co-expression system used for P450 monooxygenase activity assay (Chang et al., 2007), the ORF of either GhCPR1 or GhCPR2 was inserted into *Bgl*II-*Xho*I sites of pETDUET-1 (Novagen, Madison, USA), and the native ORF of CYP73A25 into the *Sal*I-*Not*I sites of either empty pETDUET-1 or pETDUET-1 harboring either GhCPR, respectively. The constructs were transferred into *E. coli* BL21 (DE3), and expressed in Terrific Broth medium as described above. The bacterial cells were collected by centrifugation at 4000 rpm for 5 min at 4 °C, washed twice with 4 °C MOPS buffer, and resuspended in the same buffer containing 10 μg mL^{-1} lysozyme, kept at 4 °C for 30 min. After sonication on ice, the cell lysate was centrifuged at 10,000 rpm for 2 min, and the supernatant was used for P450 activity assay. Expression of CYP73A25 was confirmed by Western blot using anti-His antibody, and the presence of GhCPR protein was confirmed by cytochrome *c* reducing activity assay.

The proteins concentration was determined using Quick Start Bradford Protein Assay kit (Bio-Rad, USA). The FMN and FAD contents were determined according to the method of Faeder and Siegel (1973).

5.5. Enzyme assays

Activities of CPRs were assayed as described (Benveniste et al., 1986). Reduction of cytochrome *c* was monitored by the increase of absorbance at 550 nm, at 25 °C, in 50 mM Tris buffer, pH 7.4, containing 20 μM cytochrome *c* and 20 μM NADPH. A molar absorption coefficient of 21 mM^{-1} cm^{-1} for equine heart cytochrome *c* was used for quantification. The reduction of DCPIP was monitored at 600 nm (20.6 mM^{-1} cm^{-1}), $K_3Fe(CN)_6$ at 424 nm (1.02 mM^{-1} cm^{-1}).

For measuring kinetic parameters, recombinant GhCPR1 and GhCPR2 proteins were assayed in 50 mM Tris buffer at 25 °C, pH 7.4, respectively. To determine kinetic parameters for cytochrome *c*, 100 μM NADPH was added to the reaction mixtures containing varying concentrations of cytochrome *c*. The kinetic parameters for NADPH was measured by using 100 μM cytochrome *c* with varying NADPH concentrations. Values were obtained by Lineweaver–Burke plot analysis with GraphPad Prism 5 software.

Cinnamic acid 4-hydroxylase activity was assayed in 50 mM phosphate buffer, pH 7.4, containing 1 mM *trans*-cinnamic acid. The reaction was started by adding 2 mM NADPH. After incubation at 30 °C for 30 min, equal volume of MeOH was added into the reaction mixture and formation of *p*-coumaric acid was determined by HPLC (Agilent 1100) using an Agilent ZORBAX Eclipse XDB-C18 analytical column (150 mm × 4.6 mm, 5 μm), based on a published method (Urban et al., 1994).

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References

Benveniste, I., Gabriac, B., Durst, F., 1986. Purification and characterization of the NADPH-cytochrome P450 (cytochrome c) reductase from higher-plant microsomal fraction. Biochem. J. 235, 365–373.
Benveniste, I., Lesot, A., Hasenfratz, M.P., Kochs, G., Durst, F., 1991. Multiple forms of NADPH-cytochrome P450 reductase in higher plants. Biochem. Biophys. Res. Commun. 177, 105–112.
Brankova, L., Ivanov, S., Alexieva, V., 2007. The induction of microsomal NADPH:cytochrome P450 and NADH:cytochrome b(5) reductases by long-term salt treatment of cotton (*Gossypium hirsutum* L.) and bean (*Phaseolus vulgaris* L.) plants. Plant Physiol. Biochem. 45, 691–695.
Chang, M.C., Eachus, R.A., Trieu, W., Ro, D.K., Keasling, J.D., 2007. Engineering *Escherichia coli* for production of functionalized terpenoids using plant P450s. Nat. Chem. Biol. 3, 274–277.
Chapple, C., 1998. Molecular-genetic analysis of plant cytochrome P450-dependent monooxygenases. Ann. Rev. Plant Physiol. Plant Mol. Biol. 49, 311–343.
Chen, X.Y., Chen, Y., Heinstein, P., Davisson, V.J., 1995. Cloning, expression, and characterization of (+)-δ-cadinene synthase: a catalyst for cotton phytoalexin biosynthesis. Arch. Biochem. Biophys. 324, 255–266.
Choi, D., Ward, B.L., Bostock, R.M., 1992. Differential induction and suppression of potato 3-hydroxy-3-methylglutaryl coenzyme A reductase genes in response to *Phytophthora infestans* and to its elicitor arachidonic acid. Plant Cell 4, 1333–1344.
Dohr, O., Paine, M.J., Friedberg, T., Roberts, G.C., Wolf, C.R., 2001. Engineering of a functional human NADH-dependent cytochrome P450 system. Proc. Natl. Acad. Sci. USA 98, 81–86.
Essenberg, M., Grover Jr., P.B., Cover, E.C., 1990. Accumulation of antibacterial sesquiterpenoids in bacterially inoculated Gossypium leaves and cotyledons. Phytochemistry 29, 3107–3113.
Faeder, E.J., Siegel, L.M., 1973. A rapid micromethod for determination of FMN and FAD in mixtures. Anal. Biochem. 53, 332–336.
Feldmann, K.A., 2001. Cytochrome P450s as genes for crop improvement. Curr. Opin. Plant Biol. 4, 162–167.
Friesen, J.A., Rodwell, V.W., 2004. The 3-hydroxy-3-methylglutaryl coenzyme-A (HMG-CoA) reductases. Genome Biol. 5, 248.
Fukuchi-Mizutani, M., Mizutani, M., Tanaka, Y., Kusumi, T., Ohta, D., 1999. Microsomal electron transfer in higher plants: cloning and heterologous expression of NADH-cytochrome b5 reductase from Arabidopsis. Plant Physiol. 119, 353–362.
Ha, S.H., Lee, S.W., Kim, Y.M., Hwang, Y.S., 2001. Molecular characterization of HMG2 gene encoding a 3-hydroxy-methylglutaryl-CoA reductase in rice. Mol. Cells 11, 295–302.
Hull, A.K., Celenza, J.L., 2000. Bacterial expression and purification of the Arabidopsis NADPH-cytochrome P450 reductase ATR2. Protein Expr. Purif. 18, 310–315.
Ilan, Z., Ilan, R., Cinti, D.L., 1981. Evidence for a new physiological role of hepatic NADPH:ferricytochrome (P-450) oxidoreductase. Direct electron input to the microsomal fatty acid chain elongation system. J. Biol. Chem. 256, 10066–10072.
Kim, B.R., Nam, H.Y., Kim, S.U., Pai, T., Chang, Y.J., 2004. Reverse transcription quantitative-PCR of three genes with high homology encoding 3-hydroxy-methylglutaryl-CoA reductase in rice. Biotechnol. Lett. 26, 985–988.
Koopmann, E., Hahlbrock, K., 1997. Differentially regulated NADPH:cytochrome P450 oxidoreductases in parsley. Proc. Natl. Acad. Sci. USA 94, 14954–14959.
Kumar, S., Nei, M., Dudley, J., Tamura, K., 2008. MEGA: a biologist-centric software for evolutionary analysis of DNA and protein sequences. Brief Bioinform. 9, 299–306.
Kunic, B., Truan, G., Breskvar, K., Pompon, D., 2001. Functional cloning, based on azole resistance in *Saccharomyces cerevisiae*, and characterization of *Rhizopus nigricans* redox carriers that are differentially involved in the P450-dependent response to progesterone stress. Mol. Genet. Genomics 265, 930–940.
Lah, L., Krasevec, N., Trontelj, P., Komel, R., 2008. High diversity and complex evolution of fungal cytochrome P450 reductase: cytochrome P450 systems. Fungal Genet. Biol. 45, 446–458.

Lesuisse, E., Casteras-Simon, M., Labbe, P., 1997. Cytochrome P-450 reductase is responsible for the ferrireductase activity associated with isolated plasma membranes of *Saccharomyces cerevisiae*. FEMS Microbiol. Lett. 156, 147–152.

Liang, W., Tan, X., Chen, X., Hashimoto, T., Yamada, Y., Heinstein, P., 2000. Isolation of a (+)-δ-cadinene synthase gene CAD1-A and analysis of its expression pattern in seedlings of *Gossypium arboreum* L.. Sci. China Ser. C: Life Sci. 43, 245–253.

Liu, C.J., Heinstein, P., Chen, X.Y., 1999. Expression pattern of genes encoding farnesyl diphosphate synthase and sesquiterpene cyclase in cotton suspension-cultured cells treated with fungal elicitors. Mol. Plant Microbe Interact. 12, 1095–1104.

Luo, P., Wang, G., Chen, X., 2001a. Isolation and expression analysis of two cDNAs encoding C4H homologues from *Gossypium arboreum*. Acta Bot. Sin. 43, 77–81.

Luo, P., Wang, Y.H., Wang, G.D., Essenberg, M., Chen, X.Y., 2001b. Molecular cloning and functional identification of (+)-δ-cadinene-8-hydroxylase, a cytochrome P450 mono-oxygenase (CYP706B1) of cotton sesquiterpene biosynthesis. Plant J. 28, 95–104.

Malonek, S., Rojas, M.C., Hedden, P., Gaskin, P., Hopkins, P., Tudzynski, B., 2004. The NADPH-cytochrome P450 reductase gene from *Gibberella fujikuroi* is essential for gibberellin biosynthesis. J. Biol. Chem. 279, 25075–25084.

Mao, Y.B., Cai, W.J., Wang, J.W., Hong, G.J., Tao, X.Y., Wang, L.J., Huang, Y.P., Chen, X.Y., 2007. Silencing a cotton bollworm P450 monooxygenase gene by plant-mediated RNAi impairs larval tolerance of gossypol. Nat. Biotechnol. 25, 1307–1313.

Meijer, A.H., Lopes Cardoso, M.I., Voskuilen, J.T., de Waal, A., Verpoorte, R., Hoge, J.H., 1993. Isolation and characterization of a cDNA clone from *Catharanthus roseus* encoding NADPH:cytochrome P-450 reductase, an enzyme essential for reactions catalysed by cytochrome P-450 mono-oxygenases in plants. Plant J. 4, 47–60.

Mizutani, M., Ohta, D., 1998. Two isoforms of NADPH:cytochrome P450 reductase in *Arabidopsis thaliana*. Gene structure, heterologous expression in insect cells, and differential regulation. Plant Physiol. 116, 357–367.

Murataliev, M.B., Arino, A., Guzov, V.M., Feyereisen, R., 1999. Kinetic mechanism of cytochrome P450 reductase from the house fly (*Musca domestica*). Insect Biochem. Mol. Biol. 29, 233–242.

Murataliev, M.B., Feyereisen, R., 2000. Interaction of NADP(H) with oxidized and reduced P450 reductase during catalysis. Studies with nucleotide analogues. Biochemistry 39, 5066–5074.

Nelson, A.J., Doerner, P.W., Zhu, Q., Lamb, C.J., 1994. Isolation of a monocot 3-hydroxy-3-methylglutaryl coenzyme A reductase gene that is elicitor-inducible. Plant Mol. Biol. 25, 401–412.

Nelson, D.R., Schuler, M.A., Paquette, S.M., Werck-Reichhart, D., Bak, S., 2004. Comparative genomics of rice and Arabidopsis. Analysis of 727 cytochrome P450 genes and pseudogenes from a monocot and a dicot. Plant Physiol. 135, 756–772.

O'Donnell, V.B., Smith, G.C., Jones, O.T., 1994. Involvement of phenyl radicals in iodonium inhibition of flavoenzymes. Mol. Pharmacol. 46, 778–785.

Portal, P., Villamil, S.F., Alonso, G.D., De Vas, M.G., Flawia, M.M., Torres, H.N., Paveto, C., 2008. Multiple NADPH-cytochrome P450 reductases from *Trypanosoma cruzi* suggested role on drug resistance. Mol. Biochem. Parasitol. 160, 42–51.

Porter, T.D., Beck, T.W., Kasper, C.B., 1990. NADPH-cytochrome P-450 oxidoreductase gene organization correlates with structural domains of the protein. Biochemistry 29, 9814–9818.

Reichhart, D., Salaun, J.P., Benveniste, I., Durst, F., 1980. Time course of induction of cytochrome P-450, NADPH-cytochrome c reductase, and cinnamic acid hydroxylase by phenobarbital, ethanol, herbicides, and manganese in higher plant microsomes. Plant Physiol. 66, 600–604.

Ro, D.K., Ehlting, J., Douglas, C.J., 2002. Cloning, functional expression, and subcellular localization of multiple NADPH-cytochrome P450 reductases from hybrid poplar. Plant Physiol. 130, 1837–1851.

Rosco, A., Pauli, H.H., Priesner, W., Kutchan, T.M., 1997. Cloning and heterologous expression of NADPH-cytochrome P450 reductases from the Papaveraceae. Arch. Biochem. Biophys. 348, 369–377.

Schuler, M.A., Werck-Reichhart, D., 2003. Functional genomics of P450s. Ann. Rev. Plant Biol. 54, 629–667.

Scotto-Lavino, E., Du, G., Frohman, M.A., 2006. 5′ end cDNA amplification using classic RACE. Nat. Protoc. 1, 2555–2562.

Shet, M.S., Sathasivan, K., Arlotto, M.A., Mehdy, M.C., Estabrook, R.W., 1993. Purification, characterization, and cDNA cloning of an NADPH-cytochrome P450 reductase from mung bean. Proc. Natl. Acad. Sci. USA 90, 2890–2894.

Simmons, D.L., Lalley, P.A., Kasper, C.B., 1985. Chromosomal assignments of genes coding for components of the mixed-function oxidase system in mice. Genetic localization of the cytochrome P-450PCN and P-450PB gene families and the nadph-cytochrome P-450 oxidoreductase and epoxide hydratase genes. J. Biol. Chem. 260, 515–521.

Sunilkumar, G., Campbell, L.M., Puckhaber, L., Stipanovic, R.D., Rathore, K.S., 2006. Engineering cottonseed for use in human nutrition by tissue-specific reduction of toxic gossypol. Proc. Natl. Acad. Sci. USA 103, 18054–18059.

Tan, X.P., Liang, W.Q., Liu, C.J., Luo, P., Heinstein, P., Chen, X.Y., 2000. Expression pattern of (+)-δ-cadinene synthase genes and biosynthesis of sesquiterpene aldehydes in plants of *Gossypium arboreum* L.. Planta 210, 644–651.

Townsend, B.J., Llewellyn, D.J., 2007. Reduced terpene levels in cottonseed add food to fiber. Trends Biotechnol. 25, 239–241.

Urban, P., Mignotte, C., Kazmaier, M., Delorme, F., Pompon, D., 1997. Cloning, yeast expression, and characterization of the coupling of two distantly related *Arabidopsis thaliana* NADPH-cytochrome P450 reductases with P450 CYP73A5. J. Biol. Chem. 272, 19176–19186.

Urban, P., Werck-Reichhart, D., Teutsch, H.G., Durst, F., Regnier, S., Kazmaier, M., Pompon, D., 1994. Characterization of recombinant plant cinnamate 4-hydroxylase produced in yeast. Kinetic and spectral properties of the major plant P450 of the phenylpropanoid pathway. Eur. J. Biochem. 222, 843–850.

Vermilion, J.L., Ballou, D.P., Massey, V., Coon, M.J., 1981. Separate roles for FMN and FAD in catalysis by liver microsomal NADPH-cytochrome P-450 reductase. J. Biol. Chem. 256, 266–277.

Wang, J., de Montellano, P.R., 2003. The binding sites on human heme oxygenase-1 for cytochrome p450 reductase and biliverdin reductase. J. Biol. Chem. 278, 20069–20076.

Werck-Reichhart, D., Hehn, A., Didierjean, L., 2000. Cytochromes P450 for engineering herbicide tolerance. Trends Plant Sci. 5, 116–123.