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Classification and Characterization of Putative Cytochrome P450 Genes from *Panax ginseng* C. A. Meyer

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Abstract—In plants heme containing cytochrome P450 (P450) is a superfamily of monooxygenases that catalyze the addition of one oxygen atom from O_2 into a substrate, with a substantial reduction of the other atom to water. The function of P450 families is attributed to chemical defense mechanism under terrestrial environmental conditions; several are involved in secondary and hormone metabolism. However, the evolutionary relationships of P450 genes in *Panax ginseng* remain largely unknown. In the present study, data mining methods were implemented and 116 novel putative P450 genes were identified from Expressed Sequence Tags (ESTs) of a ginseng database. These genes were classified into four clans and 22 families by sequence similarity conducted at amino acid level. The representative putative P450 sequences of *P. ginseng* and known P450 family from other plants were used to construct a phylogenetic tree. By comparing with other genomes, we found that most of the P450 genes from *P. ginseng* can be found in other dicot species. Depending on P450 family functions, seven P450 genes were selected, and for that organ specific expression, abiotic, and biotic studies were performed by quantitative reverse transcriptase-polymerase chain reaction. Different genes were found to be expressed differently in different organs. Biotic stress and abiotic stress transcript level was regulated diversely, and upregulation of P450 genes indicated the involvement of certain genes under stress conditions. The upregulation of the P450 genes under methyl jasmonate and fungal stress justifies the involvement of specific genes in secondary metabolite biosynthesis. Our results provide a foundation for further elucidating the actual function and role of P450 involved in various biochemical pathways in *P. ginseng*.

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Key words: abiotic, biotic, ESTs, gene expression, *in silico*, *Panax ginseng*, secondary metabolites

Cytochrome P450 (P450) constitutes a group of heme-thiolate proteins that catalyze numerous biosynthetic and xenobiotic pathways. P450 was first identified in 1958 due to its spectral properties [1]. The name P450 was given for the absorption of carbon monoxide bound form at 450 nm, and these proteins belong to one of the largest superfamilies of enzyme proteins that exist in all living organisms [2]. Plant P450s participate in a wide range of biochemical pathways to produce primary and secondary metabolites such as phenylpropanoids, alkaloids, terpenoids, lipids, cyanogenic glycosides and glucosinolates, plant hormones, signaling molecules, and structural polymers [3]. P450 superfamily genes are classified and subdivided by following nomenclature rules that are based on amino acid identity, phylogenetic analysis, and gene organization [4]. CYP is the root symbol followed by a number of families (>40% amino acid identity), a letter of subfamilies (>55% amino acid identity), and followed by number of proteins [5]. Genome sequencing projects have revealed that there is large number of plant P450 genes, and more than 3000 P450 sequences have been stored in databases. The number of P450 genes has been estimated in plant genomes, and up to 1% of total gene annotations of each species have been

Abbreviations: CDART, conserved domain architecture retrieval tool; EST, Expressed Sequence Tags; MeJA, methyl jasmonate; P450, cytochrome P450; qRT-PCR, quantitative real time reverse transcriptase-polymerase chain reaction.

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found [6] indicating that P450 is one of the largest gene superfamilies. In plants, P450 is generally classified into the A-type and non-A-type [7]. A-type P450s originated from a single ancestor and have been found to play important roles in synthesis of secondary metabolites and natural compounds or plant specific compounds, in order to adapt biotic and abiotic stress conditions and to communicate among plants. Non-A-type P450s are a divergent group of sequences that constitute an individual clan, often showing similarity to non-A-type plant P450s [8]. Previous studies have shown that a few cytochrome P450 families are responsible for defense-related mechanisms. One such example (CYP71D8 and CYP71D9) is found in soybeans, which is induced and produces stress-inducible metabolites (volatile compounds like terpenoids) [9].

Korean ginseng (*Panax ginseng* C. A. Meyer) is a typical medicinal plant and has been used as a traditional medicine since ancient times. The major active component of ginseng roots is ginsenoside, a triterpenoid saponin. Saponins have been shown to have many beneficial bioactive effects on human health, such as antitumor, anti-stress, anti-aging, and also improvement of immune function. Due to its vast pharmaceutical properties, special attention has been paid to genes involved in ginsenoside biosynthesis for large-scale production. Ginsenosides are synthesized by the cyclization of 2,3-oxidosqualene via the mevalonate pathway. This cyclization occurs at the branching point leading to oleanane-type and dammarane-type ginsenosides. Dammarane-type triterpenes undergoes further modifications such as oxidation/hydroxylation, substitution, and glycosylation leading to protopanaxadiol- and protopanaxatriol-type ginsenosides. Despite commercial interest in ginsenosides, very little is known about the genes involved in the biosynthetic pathway of triterpenoid saponins [10]. Among the genes involved, cytochrome P450 is thought to play a role in hydroxylation, resulting in protopanaxadiol- and protopanaxatriol-type ginsenosides [11]. However, the candidate P450 involved in the hydroxylation process has rarely been investigated. In a preliminary study, we classified P450s in order to predict possible function of genes according to their family functions. Expressed Sequence Tags (ESTs) of a ginseng database (www.bioherbs.khu.ac.kr/ and www.bioherbs.khu.ac.kr/ggrb/) contains over 331,430 genes including ESTs of various organs like leaf, stem, flower bud, 2-, 4-, 6-, and 14-year root, hairy root, and methyl jasmonate (MeJA)-treated hairy root. From the above ESTs, we assembled 99,568 reads into contigs. Later, we collected and assembled all the cytochrome P450s from different organs into large contigs obtaining a total of 116 contigs. We identified and annotated the cytochrome P450s according to their similarities to P450s of other plants. These data allowed us to predict novel genes; which in turn provided the way to identify the probable function of uncharacterized P450 sequences involved in a vast array of secondary metabolite genes including terpenoid synthesis, hydroxylation of fatty acids, stress inducible genes, and in other functions.

In the present study, using *in silico* and bioinformatic tools, the putatively functional P450 encoding sequences from *P. ginseng* ESTs were identified and a few genes were characterized. The deduced amino acid sequences were used to classify the cytochrome P450 genes into respective families. Previous studies have demonstrated that ginseng cultivation takes more than four years and is therefore more likely to be affected by environmental stresses [12]. In order to evaluate the induction of P450 genes under normal and stressful conditions, organ specific expression, expression with respect to biotic stresses, and MeJA elicitor treated hairy root cultures were studied for selected cytochrome P450 (based on the family functions of other plant P450s) using quantitative real time reverse transcriptase-polymerase chain reaction (qRT-PCR). In addition, we also studied the EST derived tissue specific expression profiles for all predicted P450 sequences.

MATERIALS AND METHODS

Identification of putative P450 from the *P. ginseng* EST database. Using the pyrosequencing method, putative P450 sequences from *P. ginseng* were collected from a ginseng EST database (www.bioherbs.khu.ac.kr/ and www.bioherbs.khu.ac.kr/ggrb/) and assembled into large contigs. Each representative P450 member was used as a query sequence and searched against the BLAST P search process [13] conducted at the amino acid level. Each preferentially expressed member with an identified family was used to predict the family of putative P450 genes of *P. ginseng*. For each query, BLAST P hits were collected in a file when the deduced amino acid sequences were more than 40% identical to the corresponding query sequence. In this way all putative P450 sequences were named according to the standard system of P450 nomenclature [4]. These assigned families were recorded and used for further analysis.

Generation of the unigene dataset. The unigene dataset contains a set of non-redundant sequences composed of singlets and contigs. Generating a unigene dataset requires several steps to process the sequences. All are integrated and freely accessible online at EG assembler (http://egassembler.hgc.jp/) [14]. Data was uploaded to the server using the default parameters. The bioinformatics pipeline starts with sequence cleaning, masking of low quality sequence, removal of repetitive elements, and organelle sequences masking. The resulting sequences were used to generate contig sequences using the CAP3 software [15].

Subcellular localization and phylogenetic analysis of putative P450s. Partial or full-length EST sequence at

amino acid level was used to predict the putative subcellular localization of cytochrome P450. Predictions of subcellular localization were fulfilled using the Target P program [16] with default parameters, which are available at the center for Biological Sequence Analysis (http://www.cbs.dtu.dk/services/Target P). A family member of predicted P450 sequence and representative member of the known P450 families from other plants were used for the construction of a phylogenetic tree. The query sequence was aligned to known P450 sequences from other plants, using the ClustalX (version 1.81) program [17]. The aligned sequences were used to build a phylogenetic tree following the neighbor-joining method (NJ) using Mega 4 software [18]. The significance level of the NJ was examined by bootstrap testing with 1000 repeats. The tree was represented using Boot strap view.

Plant materials and fungal strains. The *Panax ginseng* C. A. Meyer seedlings and hairy roots were obtained from Korean Ginseng Resource Bank, South Korea. The 4-week-old ginseng seedlings were grown in *in vitro* conditions on solidified MS media (Duchefa Biochemie, The Netherlands) [19] with 3% sucrose and supplemented with gibberellic acid (30 mg/ml) at 25°C under a 16 h photoperiod. The 1-month-old subcultures of hairy root were grown in hormone-free liquid MS media (Duchefa Biochemie) with 3% sucrose at 25°C under a 16 h photoperiod [20].

The fungal strains *Botrytis cinerea* (KACC 41298), *Colletotrichum gloeosporioides* (KACC 40003), *Rhizoctonia solani* (KACC 40101), and *Pythium ultimum* (KACC 41062) were obtained from Korean Agricultural Culture Collection (KACC), South Korea. We particularly focused on these strains because these fungal strains causes damping-off, rusty rot, and fruit and leaf blight diseases in ginseng [21].

Organ specific expression and MeJA induced expression. We studied the expression patterns of P450 genes in 4-year-old field samples of *P. ginseng*. After harvest, organs were separated (leaf, stem, flower bud, rhizome, and main root), washed with distilled water, dried with filter paper, and immediately frozen in liquid nitrogen and stored at −70°C until expression studies were performed. For MeJA treatments, hairy root cultures placed in liquid MS [20] that had been subcultured for one month grown at 25°C under a 16-h photoperiod, 50 μM MeJA were treated and samples were collected at 4, 8, 12, 24 and 48 h intervals. Control hairy roots were also grown for one month in liquid MS, at 25°C under a 16 h photoperiod. The stressed plant materials from all completed treatments were immediately frozen in liquid nitrogen and stored at −70°C until required.

Biotic stress induced expression. The *P. ginseng* C. A. Meyer seeds used in the present study were obtained from the Korean Ginseng Resource Bank, South Korea. The 4-week-old ginseng seedlings germinated in *in vitro* conditions on solidified MS media [19] with 3% sucrose supplemented with gibberellic acid (30 mg/ml) at 25°C under a 16 h photoperiod were used for the expression studies. Biotic stress treatment was given to 1-month-old ginseng seedlings by following the method described previously [22]. Untreated seedlings were used as control. After fungal treatment, seedlings were harvested at 6, 24, 48, and 72 h intervals.

RNA extraction, cDNA synthesis, and qRT-PCR analysis. RNA was extracted from *P. ginseng* organs that were subjected to all experimental treatments using the RNeasy kit (Qiagen, USA) according to the manufacturer's instructions. The quality and concentration of RNA was measured using a spectrophotometer (GE Nanovalue, USA). To obtain the first strand of cDNA, 1 μg of total RNA was used, and cDNA was synthesized using a Power cDNA kit (Invitrogen, USA) following the manufacturer's instructions. We performed qRT-PCR using a real-time rotary analyzer (Rotor-Gene 6000; Corbett Life Science, Australia) using 100 ng of cDNA in a 10 μl reaction volume, using SYBR® Green SensiMix Plus Master Mix (Quantace, England), and gene specific primers ware listed in Table 1. The housekeeping gene encoding actin protein was used as a standard for all samples. For all transcript analysis of stress treated samples, untreated samples were used as negative control. The melting point analysis of PCR products was carried out, which resulted in a single peak, indicating the presence of a single PCR product amplification. The melting temperature varies from 78.6-80°C with no clear differences between the treatments. The PCR conditions for each 40 cycles were 95°C for 10 sec, 60°C for 10 sec, and 72°C for 20 sec. Fluorescence was detected and measured in the real-time Reverse Transcriptase PCR thermocycler, and its geometric increase of the fluorescence corresponding to an exponential increase of the product was used to determine the threshold cycle (Ct) in each reaction using the formula $2^{-\Delta\Delta}$Ct. All real time Reverse Transcriptase PCR experiments were performed in triplicates.

RESULTS AND DISCUSSION

Identification of putative P450 genes from ginseng EST database. We constructed a cloned cDNA library from various organs, namely leaf, stem, flower bud, hairy root cultures, embryogenic callus, MeJA-treated hairy root cultures, 2-, 4-, 6-, and 14-year-old root samples. A unigene dataset was generated using the EG bioinformatics pipeline. Over 331,430 EST sequences were used, and further we removed non-informative sequence stretches such as retroelements, LTR elements, TY1/Copia, Gypsy/DIRST1, Gypsy/DIRST1, DNA transposons, Hodo-Activator, and MuDR-IS905 elements [14]. The CAP3 embedded with EG returned over 1780 contigs as partial and full-length sequences of *P. ginseng*. All analyses were performed with default parameters. We retrieved

Table 1. List of P450 gene specific primer sequences used for the present study

No.	P450 gene	Accession No./EST No.	Specific primer sequence (5′-3′)
1	CYP71D184	DC0_contig 44	**F-** TGGTCGGATCCAAACCGAGTTTA **R-** ATATCAACAGGAGCATCGGACG
2	CYP71A50U	DC0_contig 107	**F-** TGCAAGCTGAGGTGAGAGAAATAG **R-** TCTTGCAATAGCCCAAGCATTG
3	CYP704G12	DC0_contig 17	**F-** GGGAGCAGATGAAAAATGGAAAGC **R-** GTGCCACCTTTTCTTGAACTAAAGG
4	CYP82C22	DC0_contig 22	**F-** ATCCACGTTGGCAAGGATAGACA **R-** ATATCAACAGGAGCATCGGACG
5	CYP716A42	DC0_contig 37	**F-** CCTGTTGGCTGCTCTTTAGCAT **R-** AAGATCCTTTCGAGTCTGTAAT
6	CYP96A43	DC05014_14_F07_059	**F-** CATTGTGTGAAGCCATGAGGCTAT **R-** CCATCTTTCTGGCTTGAACTCCAA
7	CYP736B64	BAD15331.1	**F-** TCTGCAATATTAAACCTTCCAGCC **R-** AATTCCTTTGTGGGGATGAGAAGG
8	Actin	DC03003B12	**F-** CGTGATCTTACAGATAGCTTGATGA **R-** AGAGAAGCTAAGATTGATCCTCC

all putative P450 sequences and obtained a total of 116 sequences. All the retrieved EST sequences are available at (www.bioherbs.khu.ac.kr/ and www.bioherbs.khu.ac.kr/ggrb/). A BLAST search for the putative P450 sequences was conducted at the amino acid level. Similarity hits for the query sequence were listed by the BLAST P program. When the query sequence was more than 40% similar to another known plant P450 family, the same family and subfamily name were given to the query sequence based on standard system of nomenclature [4]. In this way, we identified 116 putative P450 genes from EST database and assigned names to all putative P450 sequences. All sequences analyzed in this study possessed the typical P450 domain signature predicted by the CDART program (http://www.ncbi.nlm.nih.gov/guide/sequence-analysis/). The search criteria that only contigs containing P450 domain were selected may cause some partial sequences to be left out. We classified 116 P450 sequences into four clans and 23 different families, indicating that these P450 genes always exist as a superfamily in *P. ginseng*, as in other plants.

Various metabolic pathways (cutin biosynthesis, lignin pathways, signaling pathways for defense related response, vascular systems, flower color, repel toxins to pathogens and herbivores) observed in plants require oxygen building complex molecules. P450 is thought to be involved in all these pathways due to its involvement in oxygenation and hydroxylation. As *P. ginseng* is considered, ginsenoside is one of the most important active secondary metabolites. It is believed that hydroxylation of dammarenediol and protopanaxadiol is catalyzed by P450. In order to predict the possible P450 genes or family involved in the ginsenoside pathway and in other metabolic pathways, comparison analysis was carried out. While comparing with a few genomes (*Arabidopsis*, *Medicago truncatula*, rice, soybean, and mosses), the *P. ginseng* clan differs from all other clans by showing variance in number of clans. However, full genome sequence of *P. ginseng* has never been reported. For the first time, we identified P450 sequences from *P. ginseng* and classified genes according to family and subfamily. We found that about half of the clan could be identified after comparisons to a few other plant genomes. After comparison, interestingly, we found that almost all the clans and families identified in *P. ginseng* are also present in *M. truncatula*, *Arabidopsis*, and rice. The CYP724 family is present in *P. ginseng* but absent in *M. truncatula* and moss. However it is present in rice, *Arabidopsis*, and soybean. CYP724B1 is involved in brassinosteroid biosynthesis [23], which is involved in biosynthesis of general compounds and therefore is present in all species. The CYP716 family is present in *P. ginseng*, soybean, *Arabidopsis*, and *M. truncatula* but absent in rice. The CYP716 family is involved in diterpenoid metabolism [24]. The CYP92 family is absent in *Arabidopsis* but present in *P. ginseng*, *M. truncatula*, and soybean. The present analysis and previous published data indicate that the CYP92A subfamily exists in all higher plants and is found to be involved in brassinosteroid biosynthesis (BR) [25]. The CYP736 family is present in *P. ginseng*, *M. truncatula*, and soybean and absent in

Arabidopsis, rice, and moss. It find noteworthy that half of the P450 sequences were identified in *P. ginseng* comparing to *Arabidopsis*, which contain 246 genes, 356 genes in rice, and 332 genes in soybean and interestingly when comparing with *Panax notoginseng* P450 sequences it was shown that almost 80-90% of P450 genes were identified [26]. The variation in P450 sequences from species to species indicates that some genes are specific to certain plants, which is further confirmed by comparing *Arabidopsis* and rice, which shared only two thirds of the genes [6]. The CYP82 family is absent in rice, where as it is present in *P. ginseng*, *M. truncatula*, *Arabidopsis*, and soybean. This indicates that the CYP82 family is specific to dicots. The CYP723 and CYP93 family exist only in rice and are absent in dicot plants (*P. ginseng*, *Arabidopsis*, *M. truncatula*, and soybean), showing that these families are specific to monocots. We therefore determined that plant P450 diversification occurred before the divergence of monocots and dicots. Since the genome project was not fully elucidated, some genes existing in *P. ginseng* may be missing in the EST database.

P450s are membrane-localized proteins, mostly anchored on the cytoplasmic surface of endoplasmic reticulum by a hydrophobic peptide present at the N-terminus, usually forming a transmembrane segment [27]. Although subcellular localization of P450 proteins is difficult, the Target P program is considered to be the best program [16] for predicting P450 locations. We found that most of the *P. ginseng* P450 genes were located in secretary pathways (endoplasmic reticulum, vacuoles, and Golgi complex) and in mitochondria. Other P450 genes could not be predicted by this program because sequences were missing the 5′ end.

Phylogenetic analysis of predicted P450 families from *P. ginseng* ESTs. The neighbor-joining method was used to construct a phylogenetic tree to find the evolutionary relationship between *P. ginseng* P450 sequences and other plant P450 sequence using the Mega 4 package (Fig. 1). Like other plant P450, *P. ginseng* P450s were classified into two different types: A-type and non-A-type. Representative members of putative P450 from each family were clustered into corresponding clans. In *P. ginseng*, we identified both A-type and non-A-type P450 genes and found that A-type genes form a single large clan (CYP71) containing more than half of the identified *P. ginseng* P450 genes that play roles in secondary metabolites biosynthesis and species specific compounds such as phenylpropanoids or isoprenoids. Non-A-type genes include the remaining three clans (CYP72, CYP85, CYP86), which often contain multi-kingdom enzymes involved in synthesis of more general compounds such as sterols or fatty acids or signaling molecules.

Here we identified for the first time four clans (CYP71, CYP72, CYP85 and CYP86) consisting of 22 families, which in turn represent subfamilies. Previous studies described that dicot species such as *Arabidopsis*, *M. truncatula*, soybean, grape, and *Poplar* contain nine clans [28]. CYP71, CYP76, CYP78, CYP84, CYP89, CYP92, CYP78, CYP736, CYP72, CYP85, CYP88, CYP90, CYP707, CYP716, CYP724, CYP94, CYP96, CYP704, CYP81, CYP82, CYP715, and CYP724 are the novel P450 families identified in *P. ginseng*. These families were found to be involved in the synthesis of various secondary metabolites and general compounds. For instance, CYP707A are responsible for abscisic acid induced stress tolerance, the CYP716A subfamily is involved in diterpenoid metabolism rather than in gibberellic acid C-13 hydroxylation [24], CYP704A functions in fatty acid ω-hydroxylase involved in sporopollenin synthesis in the *Arabidopsis* pollen [29], CYP94A is involved in long chain fatty acid hydroxylation [30], CYP98A may play a role in hydroxylation of aromatic ring of cinnamate [31], the CYP82C subfamily functions as highly responsive to stress in tobacco, pea, and soybean [32], CYP78A play a crucial role as novel mobile factor regulating organ size and cell proliferation that exist only in land plants, suggesting that it may be involved in organ growth [33], CYP86 family may be involved in fatty acid metabolism [34], CYP724B1 was involved in brassinosteroid synthesis [23], CYP88 family encodes ent-kaurenoic acid oxidase which forms ent-kaurenoic acid [35], the CYP71 family from *Nepeta racemosa* suggests possible involvement in the metabolism of isoprenoids [36], CYP72A subfamily may possibly be involved in the biosynthesis of monoterpenes or indole alkaloids [37], and CYP90B subfamily are related to brassinosteroid biosynthesis [38].

While examining A-type and non-A-type cytochrome P450s, CYP71 is the largest family with 22 A-type subfamilies, and the CYP82 family is the most expanded family in non-A-type, with 14 subfamilies (Table 2). We found that the CYP71 clan is more divergent when compared to other P450 clans and is found to be involved in synthesis of species-specific compounds, whereas CYP85, CYP86, and CYP72 clans are involved in conserved functions. For example, the CYP85 clan is involved in isoprenoid metabolism, CYP86 clan in fatty acid metabolism, and CYP72 clan in plant hormone homeostasis [24]. A similar way of classification was also shown in soybean using some partial sequence of P450 genes [39]. Moreover, families that classified under CYP71 clan and CYP85 clan are the promising families involved in triterpenes and plant sterols [26].

EST derived expression of cytochrome P450. ESTs are short, unedited, randomly selected single-pass sequence reads obtained from cDNA libraries, which are also called the "poor man's genome" [40]. EST analysis offers a complete overview of the genes expressed in a certain organs and their relative expression levels [41]. We analyzed the expression profiles of P450s in many different organs (see "Materials and Methods"). Roots showed the maximum expression of P450 genes (over 29-37%)

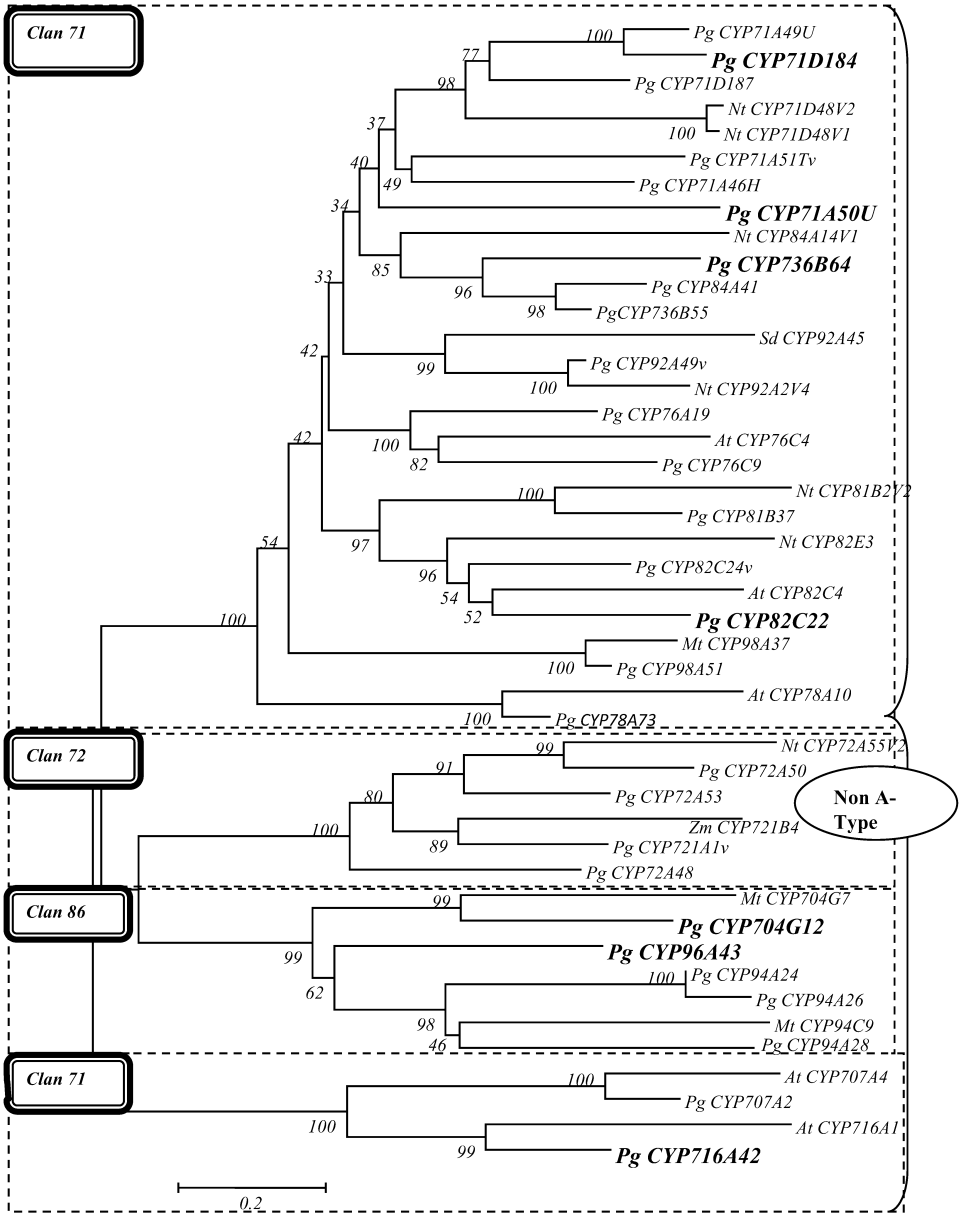

Fig. 1. Phylogenetic analysis of identified family of P450 from *P. ginseng* and other plant P450 families. The unrooted tree was constructed by the ClustalX program, the significant level of the neighbor-joining analysis was examined by bootstrap testing with 1000 repeats. The number beside the branches denotes the bootstrap values based on 1000 replications. The highlighted ones (bolded) were further selected for expression analysis. Abbreviations for other species used are as follows: *Nt*, *Nicotiana tabacum*; *At*, *Arabidopsis thaliana*; *Sd*, *Scoparia dulcis*; *Mt*, *Medicago truncatula*; *Zm*, *Zea mays*. Accession numbers and species family name included in the analysis are as follows, NM_112814 (*At*CYP707A2), EF042306 (*Nt*CYP82E3), DQ335800.1 (*Mt*CYP704G7), DQ335798.1 (*Mt*CYP94C9), DQ350334.1 (*Nt*CYP72A55v2), EU973217.1 (*Zm*CYP721B4), NM_123002 (*At*CYP716A1), DQ335791.1 (*Mt*CYP98A3), NM130117.1 (*At*CYP76C4), GU592501.1 (*Sd*CYP92A45), NM_106071 (*At*CYP88A10), DQ350347 (*Nt*CYP84A14v1), DQ350338.1 (*Nt*CYP71D48v1), DQ350337.1 (*Nt*CYP71D48v2), DQ350363.1 (*Nt*CYP72A58), XM_002869256 (*At*CYP82C4), DQ350324.1 (*Nt*CYP92A2v4).

compared to all other tested organs. We found that P450 genes were expressed in all organs except embryogenic callus. When comparing hairy root, MeJA-treated hairy root showed the highest expression indicating that some P450 genes can be highly expressed under elicitor treatment (Fig. 2).

Tissue specific expression profile of cytochrome P450 genes. A tissue specific pattern of P450s namely CYP71D184 (DC0_contig 44), CYP71A50U (DC0_contig 107), CYP704G12 (DC0_contig 12), CYP82C22 (DC0_contig 22), CYP716A42 (DC0_contig 37), CYP96A43 (DC05014_14_F07_059) and CYP736B64

Table 2. Characteristics of P450s in *P. ginseng*

Clan	Family	Name	Loc	Clan	Family	Name	Loc	Clan	Family	Name	Loc	Clan	Family	Name	Loc
71	71	CYP71A46H	–	71	76	CYP76C11	–	72	72	CYP72A55	–			CYP94A28	–
		CYP71A47H	–			CYP76C12	–			CYP72A56	–		96	CYP96A43	–
		CYP71A48H	–			CYP76C13	–			CYP72A57	–		704	CYP704A41	S
		CYP71A49U	–			CYP76H14	M			CYP72A58	–			CYP704A42	–
		CYP71A50U	–		78	CYP78A73	M			CYP72A59				CYP704G12	–
		CYP71A51Tv	–			CYP78A74	M			CYP72A60	–			CYP704G13	–
		CYP71AT52v	–			CYP78A75	M			CYP72A61	S			CYP704G14	S
		CYP71AT53v	–		84	CYP84A40	S			CYP72A62	–		81	CYP81B37	–
		CYP71A54Tv	–			CYP84A41	–			CYP72A63	S			CYP81B38	–
		CYP71D184	–			CYP84A42	–			CYP72A64				CYP81B39v	–
		CYP71D185	–		89	CYP89A69	S	85	85	CYP85A1	–			CYP81B41v	M
		CYP71D186	–		92	CYP92A48v	M		88	CYP88A30	S			CYP81D40	–
		CYP71D187	M			CYP92A49v	–		90	CYP90B20	–			CYP81E42	M
		CYP71D188	–		98	CYP98A51	–			CYP90D19	S		82	CYP82A30	–
		CYP71D189	S		736	CYP736B55	S		707	CYP707A2	–			CYP82C21p	M
		CYP71D190	–			CYP736B56	S		716	CYP716A42	–			CYP82C22	S
		CYP71D191v	–			CYP736B57	M			CYP716A43	S			CYP82C23	–
		CYP71D192v	S			CYP736B58	–			CYP716A44	–			CYP82C24v	–
		CYP71D193v	S			CYP736B60	–			CYP716A45	–			CYP82C25	S
		CYP71D194v	–			CYP736B61	–			CYP716A46	S			CYP82C26p	–
		CYP71D195v	–			CYP736B62	S			CYP716A47	S			CYP82C27	–
		CYP71D196v	–			CYP736B63	–			CYP716A48	M			CYP82C28	–
	76	CYP76A19	–			CYP736B64	M			CYP716A49	S			CYP82C29	M
		CYP76A20	–	72	72	CYP72A48	–			CYP716A50	–			CYP82C30	–
		CYP76A21	M			CYP72A49	S			CYP716A51	–			CYP82C31	–
		CYP76A22	S			CYP72A50	M		724	CYP724B16	M			CYP82C32	–
		CYP76A23	–			CYP72A51	M	86	94	CYP94A24	M			CYP82C33	–
		CYP76A24				CYP72A52	–			CYP94A25	M		715	CYP715A15	–
		CYP76C9	–			CYP72A53	S			CYP94A26	–		721	CYP721A1v	M
		CYP76C10	–			CYP72A54	M			CYP94A27	S				

Notes: Subcellular localization (Loc) was predicted for all grouped cytochrome P450: S, secretory pathway (ER); M, mitochondria; –, not predicted because the sequence 5′-end is incomplete. Prediction of subcellular localization (Loc) was based on NN scores of the Target P program using default parameters (http://www.cbs.dtu.dk/services/TargetP/).

(BAD15331.1) was determined using mRNA samples of 4-year-old organs (leaf, stem, flower bud, rhizome, main root) from field samples of *P. ginseng*. These seven P450 genes were selected based on their family functions, and tissue specific pattern was observed using qRT-PCR (Fig. 3). We found that CYP71D184 was more highly expressed in the flower bud than in all other organs. CYP71A50U was predominantly expressed in the main root, followed by the rhizome with 6.09- and 6.04-fold, respectively. CYP704G12 was equally expressed in all examined organs. The CYP82C22 family was found to be more abundant in the flower bud, with 9.01-fold expression, than other organs. CYP716A42 was most highly expressed in the leaf and flower bud, and finally CYP96A43 was most often expressed in the flower bud, average in the stem and rhizome, with the least expression in the leaf and main root. Previous study showed that expression of the dammarenediol synthase gene was higher in flower

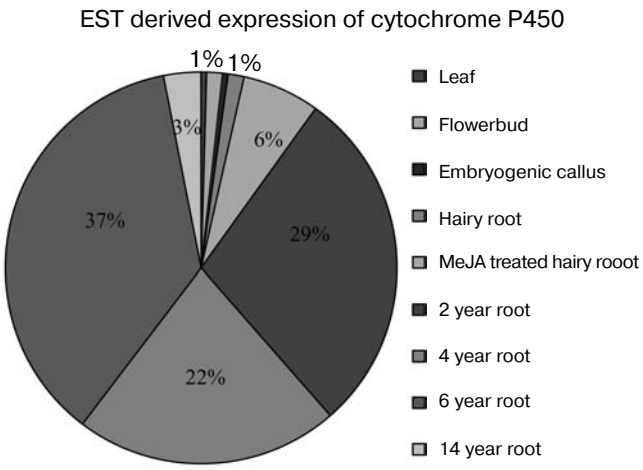

Fig. 2. Distribution of putative P450 genes in various organs of *P. ginseng* EST database.

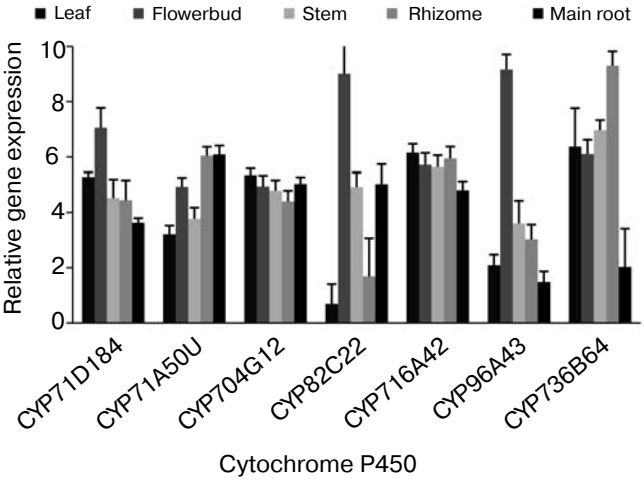

Fig. 3. Organ-specific expression of P450 families. Four-year-old field samples was collected and organs were used to quantify the expression pattern of various cytochrome P450s. Relative gene expression was observed using qRT-PCR. The Ct value for tested genes were normalized to the Ct value for β-actin and calculated relative to a calibrator using the formula $2^{-\Delta\Delta}$Ct. Data represent means ± SE for three independent replicates.

bud compared to root, leaf, and petiole of ginseng plants [42], and CYP736B64 expression was higher in rhizome with 9.3-fold, and somewhat lower in leaf, stem, and flower bud. P450 genes that are expressed highly in flower bud may be co-expressed with dammarenediol synthase gene [26]. Our results showed that CYP71D184, CYP82C22 and CYP96A43 were higher in flower bud, and CYP736B64 was abundant in flower bud, and can be the possible candidate P450 genes involved in dammarenediol type ginsenosides biosynthesis.

Fungal infection induced cytochrome P450 expression. Various environmental biotic stress conditions may also increase the transcript level of defense related and secondary metabolite related P450s [43]. Inoculation of 4-week-old *P. ginseng* seedlings with *Botrytis cinerea*, *Colletotrichum gloeosporioides*, *Rhizoctonia solani*, and *Pythium ultimum* induced P450 transcripts. The seven P450s—CYP71D184, CYP71A50U, CYP704G12, CYP82C22, CYP716A42, CYP96A43, and CYP736B64—were selected based on their family functions in order to understand the expression patterns under plant–pathogen interactions. These may be the promising candidates, which can be involved in secondary metabolite synthesis and also play a role in defense mechanisms. For example, CYP71D8 and CYP71AU1 are involved in vast array of secondary metabolite synthesis and more accumulated in infection or salicylic acid treatment [44]. Selected candidates are more likely to be expressed under stress conditions. The expression of CYP71D184 increased dramatically and reached the highest transcription level 10.9-fold at 6 h, and declined thereafter under the exposure to *B. cinerea*. During *C. gloeosporioides* infection, the expression was not significantly affected compared to the control. The mean expression value under *R. solani* infection increased initially at 6 h and reached maximum 8.4-fold at 72 h of post-treatment. *Pythium ultimum* infection attained a maximum 4.16-fold at 24 h post-treatment and decreased gradually (Fig. 4a). Induction under biotic stress condition is a general characteristic of P450 [45]. The transcript level of CYP71A50U (Fig. 4b) showed slight induction under *B. cinerea*, however it was not significantly different compared to that of control. During of *C. gloeosporioides* treatment, the transcriptome profile of CYP71A50U started at 6 h and showed highest expression at 24 h with 5.6-fold, and decreased gradually when the exposure time was prolonged. When plants were treated with *R. solani*, gene expression was highest initially at 6 h, and decreased subsequently. After *P. ultimum* infection, the transcript pattern showed increased expression between 24 and 48 h of post-treatment compared to the control.

Plants are sessile organisms, which synthesize a vast array of secondary metabolites to adapt biotic and abiotic stresses and also to interact between plant-host organisms [46]. The transcript levels of CYP704G12 under *B. cinerea* elevated, and showed a significant transcript level of 1.8-fold at 24 h and declined progressively. Under the exposure of seedlings to *C. gloeosporioides*, the expression initially showed downregulation, and gradually increased to 1.3-fold at 24 h. During the *R. solani* treatment, CYP704G12 showed an initial transcript level of 1.2-fold at 6 h, and declining thereafter. The CYP704G12 expression profile showed significant expression of 1.6-fold at 48 h, compared to that of control during *P. ultimum* infection (Fig. 4c). CYP704 is linked with fatty acid hydroxylases, and plays a major role in plant–pathogen interactions [47]. During the treatment with *B. cinerea*, CYP82C22 expression increased drastically at 6 h,

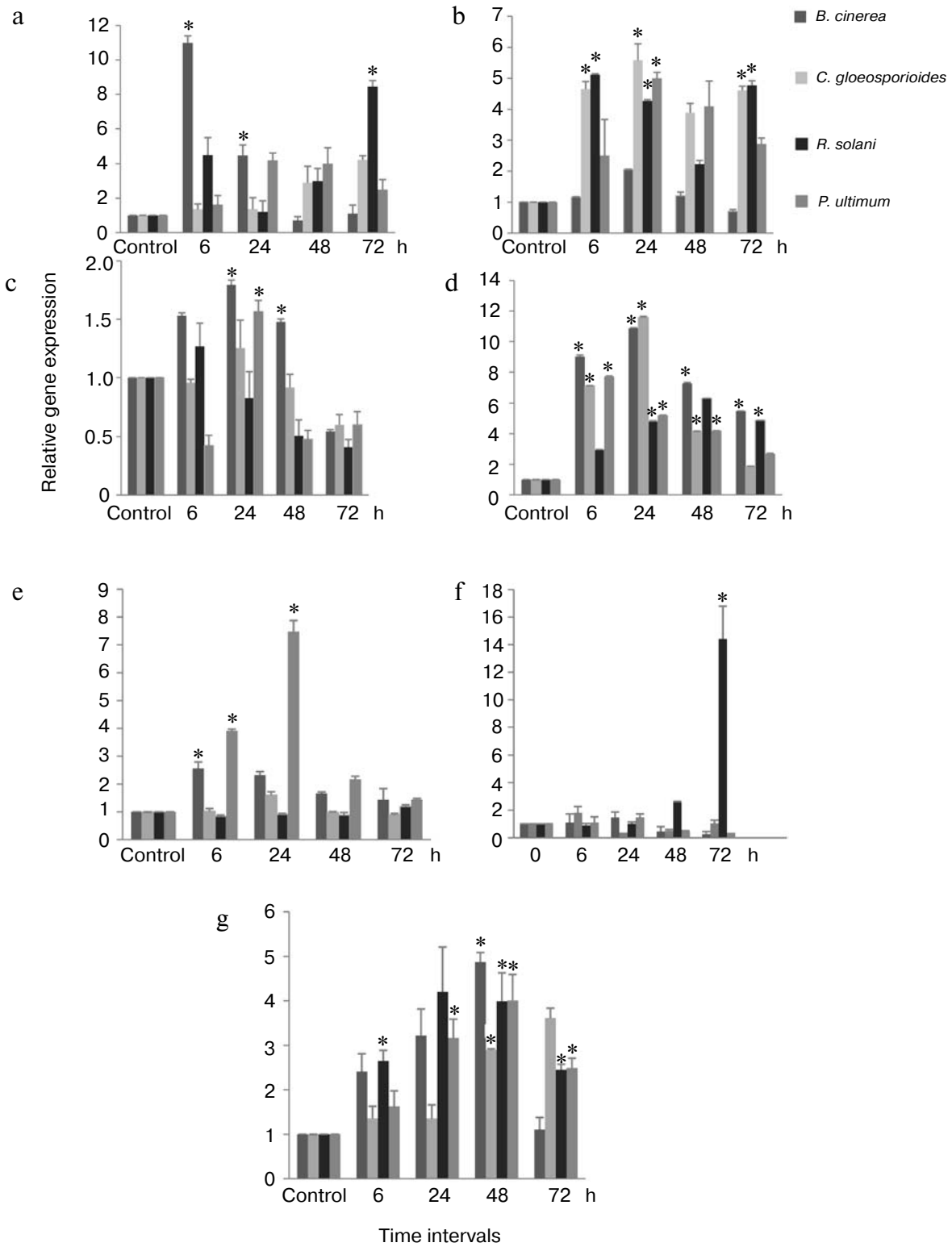

Fig. 4. Effect of biotic stresses on the expression of P450 genes. For selected P450 genes CYP71D184 (a), CYP71A50U (b), CYP704G12 (c), CYP82C22 (d), CYP716A42 (e), CYP96A43 (f), and CYP736B64 (g) biotic expression was carried out. Actively growing fungal strains were sprayed on the 4-week-old ginseng plantlets to study the expression under those stresses. The infected plantlets were collected as per the time intervals as mentioned above. The non-infected plantlets were used as control. The mRNA transcript level was detected by qRT-PCR. Data represent means ± SE for three independent replicates. Average values for treated samples are significantly different compared to the control at * $P < 0.05$ using Student's *t*-test.

reached the highest point at 24 h, and declined gradually over time. Under *C. gloeosporioides* treatment, the transcript expression began at 6 h and attained 11.6-fold at 24 h post-treatment compared to the control sample. Infection with *R. solani*, increased the mRNA level gradually, and reached a maximum 6.3-fold at 48 h post infection. *Pythium ultimum* treated seedlings showed elevated expression of CYP82C22 at 6 h and downregulated thereafter (Fig. 4d). In pea, the CYP82 family induced highly in response to UV, cold stress, wounding, and pathogen attack [48]. Similar results were reported in pepper fruit in response to fungal inoculation and wounding [49]. In gymnosperms, *Selaginella* and *Physcomitrella*, CYP716 family members are involved in diterpenoid metabolism, which are important conifer defense signals against herbivores and pathogens. Our results are in agreement with [50] which also showed defense response. The transcriptome expression of CYP716A42 showed elevated expression up to 2.6-fold at 6 h and transcript level declined after 24 h of post-treatment with *B. cinerea*. Under *C. gloeosporioides*, *R. solani*, and *P. ultimum* there was no significant expression of CYP716A42 compared to the control plantlets. Interestingly, the transcript level showed elevated expression up to 3.9-fold under *R. solani* treatment at 6 h, reaching a maximum of about 7.5-fold at 24 h and declined over the exposure time (Fig. 4e). The expression of CYP96A43 during the *B. cinerea*, *C. gloeosporioides*, and *R. solani* did not show significant increase in transcript. Surprisingly, the transcript level drastically increased under *P. ultimum* infection, reaching 15.5-fold at 72 h of post-treatment (Fig. 4f). A member of CYP96A family is currently classified under non-A-type P450 enzymes, which may use fatty acids as substrates in wax biosynthesis [6]. Cuticular waxes acts as a barrier in mediating biotic interactions with microbes [51]. Under *B. cinerea*, CYP736B64 (Fig. 4g) expression increased gradually and reached maximum at 48 h of 4.8-fold and decreased thereafter. Under *C. gloeosporioides*, the expression of CYP736B64 increased drastically and reached maximum at 72 h (3.6-fold). CYP736B64 expression under the treatment of *R. solani* reached highest at 24 h and decreased gradually. Under *P. ultimum* infection, expression of CYP736B64 showed maximum expression of 4-fold at 48 h post-treatment. Our results showed that P450 genes analyzed possessed defense related function against biotic stress except for CYP96A43, and may be involved in defense related secondary metabolite synthesis that might cause the expression of P450 transcripts.

Cytochrome P450 transcripts under MeJA treatment. Methyl jasmonate are the important signaling molecules, which play important roles in plant defense mechanism [52]. It has also been shown that MeJA activates most of the defense related proteins, namely phenyl ammonia lyase, the precursor in phenylpropanoid metabolism as well as chalone synthase, which leads to the formation of flavonoid precursors or even acts antagonistically [53, 54] and also it increases the expression of secondary metabolite genes in *P. ginseng* [10]. Upregulation of CYP71D184, CYP71A50U, and CYP82C22 genes (Fig. 5, a, b, and d) showed between 4-24 h, and reached highest at 48 h with maximum fold expression of 10.8, 45.23 and 173.8, respectively, but CYP736B64 (Fig. 5g) showed maximum expression between 12-24 h with 2.7-fold of expression. Similar results were demonstrated in roots of *P. ginseng* by MeJA treatment [55]. Expression of CYP704G12 (Fig. 5c) was found to show similar pattern in all treated and untreated samples may be of small sample number. CYP716A42 transcript (Fig. 5e) was found to be induced slightly under 4 h with a 1.62-fold increase and downregulated thereafter. However, the transcript level was not significantly affected. CYP96A43 (Fig. 5f) seemed to be downregulated, increasing gradually before reaching a maximum of 1.4-fold at 48 h of post treatment. From the above results, all the examined genes were more or less induced upon MeJA treatment, and particularly CYP71D184, CYP71A50U, CYP82C22, and CYP736B64 may be promising candidates involved in triterpenoid metabolism or defense related secondary metabolite synthesis as *P. ginseng* is considered. P450 are membrane bound proteins involved in several metabolic pathways related to defense mechanisms [56].

In conclusion, we identified 116 putative P450 genes in *P. ginseng* by a similarity search and confirmed the families of putative P450 by phylogenetic analysis. When comparing *P. ginseng* with other genomes like *Arabidopsis*, soybean, *M. truncatula*, and rice, 116 P450s sequences were identified in *P. ginseng*. These comparisons clearly showed that half of the cytochrome P450 genes were predicted in *P. ginseng*. In addition, using *in silico* and bioinformatic tools, we studied EST derived P450 expression profiles. We also studied tissue specific expression patterns in response to biotic stress and MeJA treatments. Diverse expression patterns of all seven P450 genes were observed under both biotic and MeJA treatments, interestingly P450 genes seemed to be highly expressed under MeJA treated conditions compared to biotic stress conditions. Both types of conditions may induce oxidative stress, which might have resulted in the expression of defense and secondary metabolite related transcripts.

The present study clearly shows the upregulation of defense related and secondary metabolite related P450s under various stress conditions. More importantly, P450s that might be involved in the ginsenoside pathway were studied by conducting MeJA inducibility experiments and also under biotic stress condition. Still, much research remains to be performed in order to predict the actual function of all putative P450s and to characterize the candidate P450 gene involved in the ginsenoside pathway. However, our data provide a foundation for further elucidation of the actual functions and role of cytochrome P450s in *P. ginseng*.

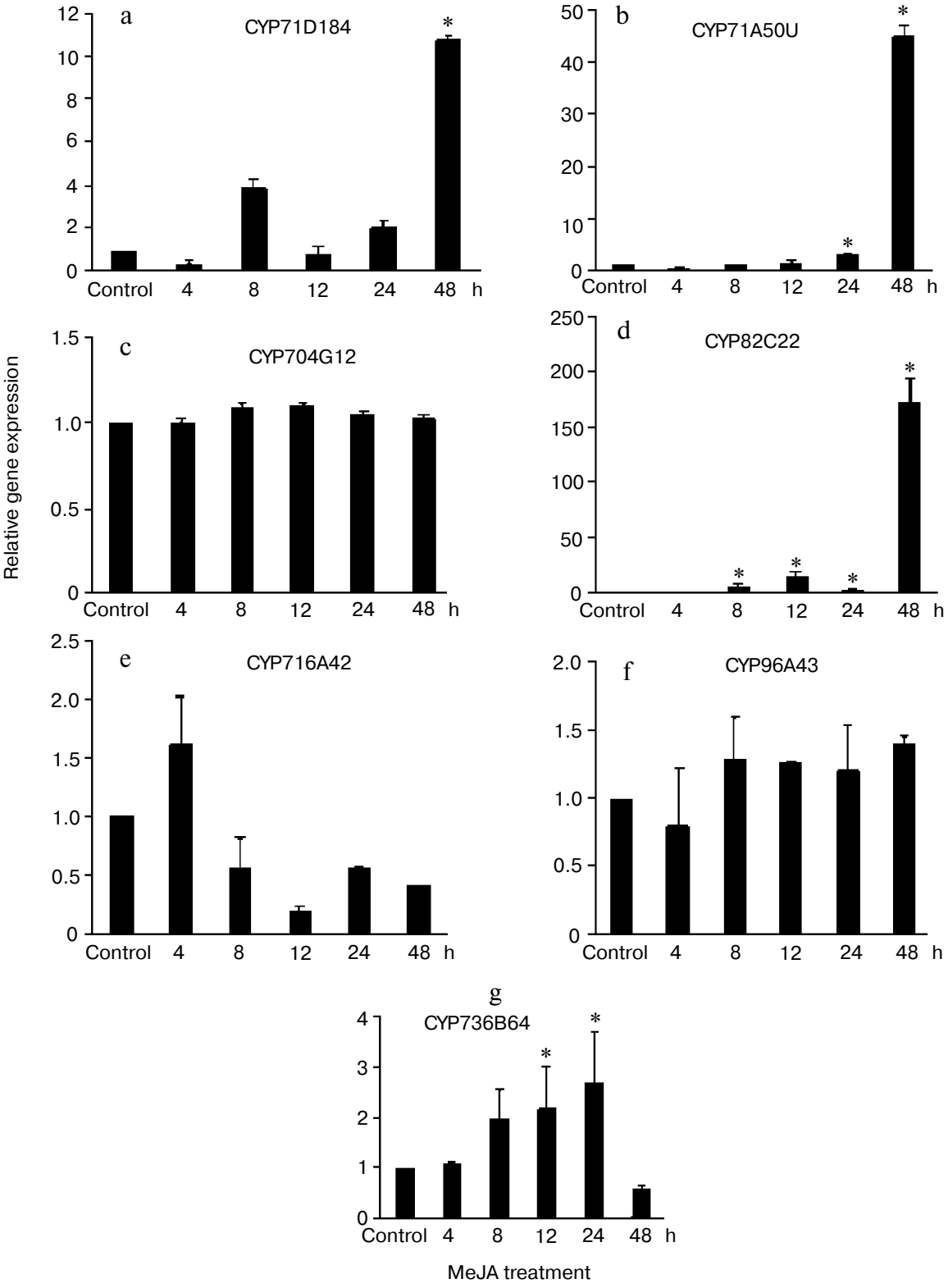

Fig. 5. Expression of P450 under 50 μM MeJA treatment in 1-month-old sub-cultured hairy root. A quantitative expression profile of selected P450 genes was performed at varying time intervals. The Ct value for each gene normalized to the Ct value for actin gene and calculated relative to a calibrator using the formula $2^{-\Delta\Delta}$Ct. Data represent the mean ± SE of three independent replicates. Average values for treated samples are significantly different compared to the control at * $P < 0.05$ using Student's *t*-test.

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