RESEARCH NOTE

Characterization of two *CYP77* gene family members related to development of ornamental organs in petunia

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Introduction

Petunia is presently one of the most important ornamental bedding plants; also known as the model plants for floral organ characteristics research (Gerats and Vandenbussche 2005; Shimamura *et al.* 2007). With comprehensive function, researches of some flower-related genes, including the MADS-box transcription factors (Theissen 2001), the plasma membrane intrinsic protein (*PIP*) genes (Azad *et al.* 2008), the α-expansin gene (*PhEXPA1*) and the xyloglucan endotransglucosylase/hydrolase (*XTH*) genes (Zenoni *et al.* 2004; Yamada *et al.* 2009), the molecular mechanism of floral organ differentiation and development have been well illustrated. However, the floral development is a complex process which contains differential gene expression regulation (Takatsuji *et al.* 1992), and genes involved in the flower growth are still largely unknown.

The cytochrome P450 (*CYP*) genes which make up one of the largest families in plants, participate in numerous reactions of the plant biological processes (Bak *et al.* 2011). The *CYP*s account for ~1% genes in some plants (Nelson *et al.* 2008), however, the functions of most *CYP*s are still unknown. In *Arabidopsis*, many *CYP*s that are associated with the flower development have been studied, like *CYP76C1* (Mizutani *et al.* 1998), *CYP77A6* (Bak *et al.* 2011; Li-Beisson *et al.* 2009), *CYP78A5* (Zondlo and Irish 1999) and three members (*CYP86A2*, *CYP86A7* and *CYP86A8*) of the *CYP86A* subfamily (Duan and Schuler 2005). As in *Petunia*, some *CYPs* expressed in the flowers have also been reported, for e.g., *CYP74C9* is upregulated during petal senescence (Xu *et al.* 2006), *CYP75A3* is involved in flower pigments (Holton *et al.* 1993), and *CYP92B1* is highly expressed in the flower buds (Petkova-Andonova *et al.* 2002).

In this study, we isolated two *CYP77* genes in petunia, *Phcyp77A1* and *Phcyp77B1*. Their specific expression patterns in different tissues, as well as their developmental regulation relationship with floral growth were analysed by the real-time quantitative polymerase chain reaction (RT-qPCR), indicating that they could play important roles in different floral development processes of petunia.

Materials and methods

RNA extraction and cDNA synthesis

Petunia hybrida 'Fantasy Red' were grown in the experimental field of Huazhong Agricultural University, Wuhan, China. The flower buds of petunia in different development stages were mixed and used for extracting RNA according to the instruction of EASYspin plant RNA extraction kit (Aidlab, Beijing, China). First-strand cDNA was synthesized from 2 μg RNA according to the instruction of TransScript One-Step gDNA Removal and cDNA Synthesis SuperMix Kit (Transgen, Beijing, China).

Isolation of Phcyp77A1 and Phcyp77B1

We constructed a suppression subtractive hybridization (SSH) cDNA library between the normal and abortive flower buds and an 801-bp cDNA fragment, RC15 (GenBank accession number JZ349629) appeared to accumulate specifically in the normal flower buds (Yue *et al.* 2013). The sequence BLAST (BLASTX) result revealed that the putative amino acid sequences encoded by RC15 was homologous to that of CYP77 proteins. According to the petunia genomic database library (http://petuniasp.sgn.cornell.edu/, unpublished), we predicted the open reading frame (ORF) of this gene (*PhCYP77B1*) which was then used as the probe, led to the detection

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Table 1. Sequences of primers used for the genes clone and RT-qPCR.

Primer name	Primer sequences (5′–3′) Forward	Reverse
Cloning primers		
Phcyp77A1	GGAAAACACAGAGAGTGAAAGAAGT	CCCACAACCATTAATTTTAGGATC
Phcyp77B1	CCCATTCTTATACTCGTAATCTCCTG	GATCGAATCACCACACCTCCT
RT-qPCR primers		
qPhcyp77A1	TGATGATTATCTTCCATTGTTGAGC	GCCCCTGATTTTCTACCTTCG
qPhcyp77B1	AGCAACGGAAACCCTAAAAGC	CATCACTAAGTGAAGCAAAGCCC
β-actin	GTTGGACTCTGGTGATGGTGTG	CCGTTCAGCAGTGGTGGTG

of another homologous (>40%) gene (*PhCYP77A1*) (Bak *et al.* 2011). The full-length ORFs of *Phcyp77A1* and *Phcyp77B1* were predicted by the FGENESH tool (http://www.softberry.com) and amplified from the mixed flower buds cDNA template with specific primers (table 1). All these primers were designed using Primer 5 software.

Sequences analysis

Sequences character computation of *Phcyp77A1* and *Phcyp-77B1* were conducted using the ProtParam tool (http://www.expasy.ch/tools/protparam.html). The amino acid sequences used in the phylogenetic tree construction were obtained from NCBI (http://www.ncbi.nlm.nih.gov/). Sequences of amino acid were aligned using the ClustalW method (www.ebi.ac.uk/clustalw). A phylogenetic tree was constructed using MEGA5 based on the neighbour-joining method with 1000 bootstrap replication (Tamura *et al.* 2011).

RT-qPCR

To analyse the expression patterns of *Phcyp77A1* and *Phcyp77B1*, samples were obtained from the flower buds at eight developmental stages (bud1 (Bl < 2 mm), bud2 (Bl 3 ± 0.5 mm), bud3 (Bl 5 ± 0.5 mm), bud4 (Bl 10 ± 0.5 mm), bud5 (Bl 15 ± 0.5 mm), bud6 (Bl 20 ± 0.5 mm), bud7 (Bl 25 ± 0.5 mm) and bud8 (Bl 35 ± 0.5 mm)), roots, fresh leaves, tender stems and four whorls of floral organs (sepals, petals, anthers and pistils) in bud2 and bud8, as well as flower petals from bud2, bud4, bud5, bud6, bud7, bud8, semiopening flowers and opening flowers respectively, were used in the RT-qPCR analysis. Upon harvesting, these materials were immediately frozen in liquid nitrogen and stored at –80°C until RNA was extracted. Total RNA was extracted and first-strand cDNA was synthesized from flower buds, anthers, and other tissues according to methods described above. The RT-qPCR was performed using an ABI 7500 Fast Sequence Detection System (PE Applied Biosystems, Foster City, USA). The RT-qPCR primers for *Phcyp77A1* and *Phcyp77B1* were designed using Primer 5 software (table 1). The *β-actin* was used as an internal control and a negative control (no template) was included in each run. Reactions were performed using the SYBR® Premix Ex TaqTM (Takara, Dalian, China). Briefly, PCR products were amplified using 1 μL template from the RT reaction mixture, 5 μL 2× SYBR® Premix Ex TaqTM, 0.5 μL each forward and reverse primer (10 μM), and water to a final volume of 20 μL. Thermal cycling conditions were programmed based on previously described methods (Yue *et al.* 2013). A melting temperature cycle was performed to confirm the presence of a single product. The RT-qPCR was performed in triplicate with RNA isolated from at least three different seedlings. The bars indicate the standard errors (S.E.).

Results and discussion

PhCYP77A1 (GenBank accession number KM055425) and *PhCYP77B1* (GenBank accession number KM055426) contain a 1548-bp ORF encoding a 515 amino acid protein and a 1533-bp ORF encoding a 510 amino acid protein, respectively. The deduced molecular mass of the mature PhCYP77A1 and PhCYP77B1 proteins are 58.6 and 57.7 kDa which coincide with all the P450 proteins that range from 45 to 62 kDa (Su and Hsu 2003). Additionally, a conserved Phe-X-X-Gly-X-Arg-X-Cys-X-Gly motif (where X could represents any amino acid) near the C-terminus and two less conserved sequences (Pro-Pro-X-Pro and Pro-Glu/Asp-Arg/His-Phe/Trp) in P450 proteins are also found in PhCYP77A1 and PhCYP77B1 (figure 1a), suggesting that they are the members of P450 proteins (Hasemann *et al.* 1995; Halkier 1996). Phylogenetic analysis shows that PhCYP77A1 is clustered closely with CYP77A subfamily and PhCYP77B1 is clustered closely with CYP77B subfamily within the five CYP77 family members of *Arabidopsis* (figure 1b). Notably, the last conserved amino acid of Phe-X-X-Gly-X-Arg-X-Cys-X-Gly motif is Ala both in Phcyp77B1 and AtCYP77B1, which are members of the CYP77B subfamily. Hence, we suggest that the conserved sequence of this motif to be modified to Phe-X-X-Gly-X-Arg-X-Cys-X-Gly/Ala.

The RT-qPCR was used to examine the spatial and temporal variation of these genes at transcript levels. The transcriptional profiles of these *PhCYP77* genes were determined in the vegetative tissues (leaves, stems and roots) and the reproductive organs (opening flowers, flower buds, sepals, petals, anthers and pistils). The highest transcript level of *PhCYP77A1* is in bud8 and *PhCYP77B1* is in bud2 when

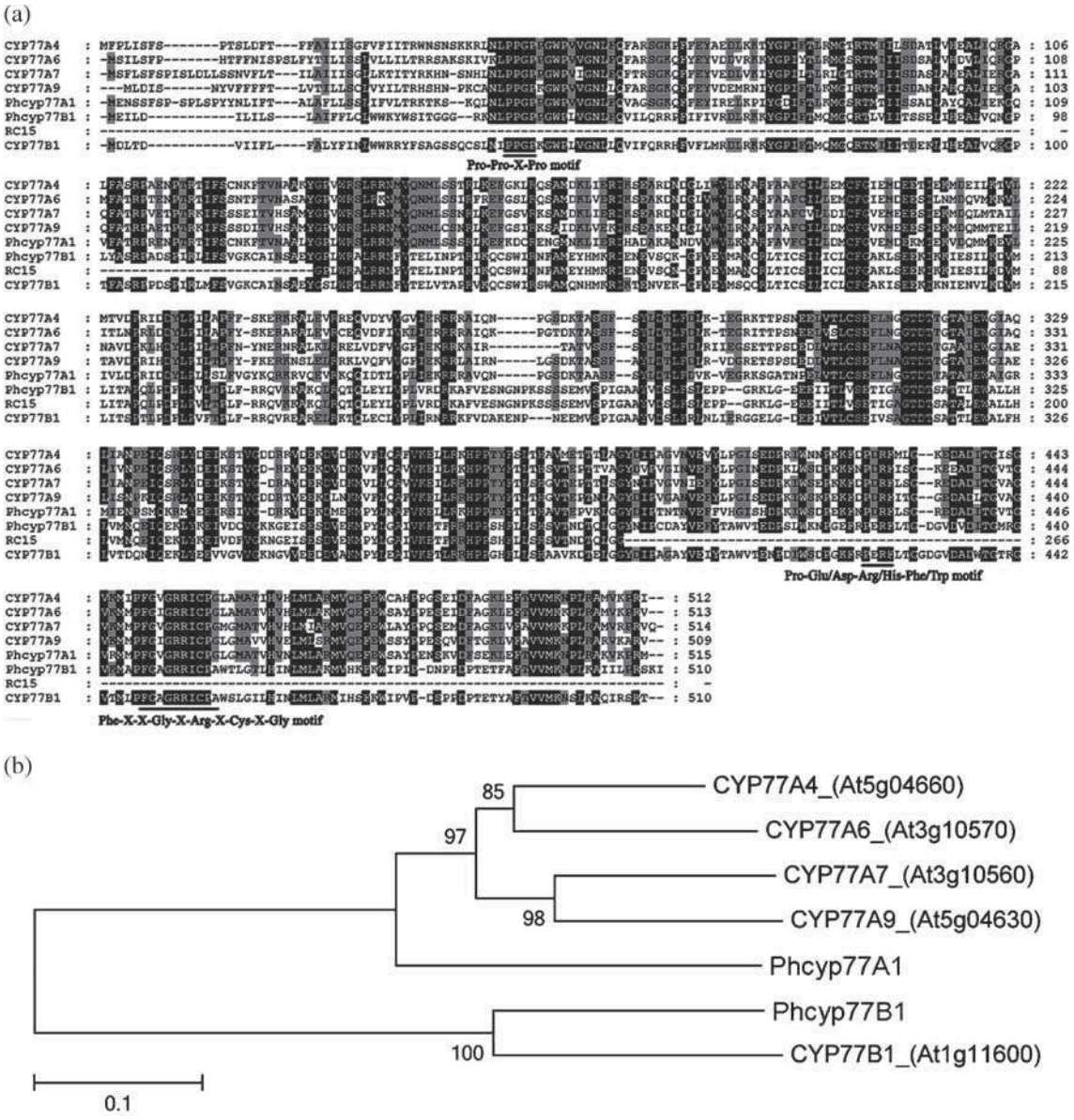

Figure 1. Sequence alignment and phylogenetic analysis of Phcyp77A1 and Phcyp77B1 proteins. (a) Alignment of Phcyp77A1 and Phcyp77B1 with reported five members of *Arabidopsis* CYP77 family. Three conserved sequences (Phe-X-X-Gly-X-Arg-X-Cys-X-Gly, Pro-Pro-X-Pro and Pro-Glu/Asp-Arg/His-Phe/Trp) are underlined by bold lines. (b) Phylogenetic tree of Phcyp77A1 and Phcyp77B1 with five reported *Arabidopsis* CYP77 family members.

compared among different tissues, implying that both are predominantly expressed in the flower buds (figure 2a). To explore the relationship between *PhCYP77* genes expression patterns and the flower bud development, the whole biological process of the flowerbud is divided into eight developmental stages. The higher transcript level of *PhCYP77A1* is in the later development stage. By contrast, the expression levels of *PhCYP77B1* are higher in the smaller flower buds, and then gradually decrease along with the development of flower buds. It is indicated that both the expression of *PhCYP77A1* and *PhCYP77B1* are development related, and their trends are reversed during the petunia flower bud development process (figure 2b). Since the development of flower buds is actually the growth and expansion in four whorls of the flower organs, the expression patterns of these genes are examined in sepals, petals, anthers and pistils. Interestingly, the utmost expression levels of these genes are both in petals (figure 2c). As the petal has remarkable changes in size and colour during the development of the flower buds, the genes highly expressed in petals could be much likely to affect the genes expression in the flower bud, and not surprisingly, the expression trends of these genes in the petals are very similar to that of the flower buds during the whole developmental stage (figure 2, b&d).

In *Arabidopsis*, the *CYP77A6*, a member of the CYP77A subfamily, is highly expressed in the petals of flower stage

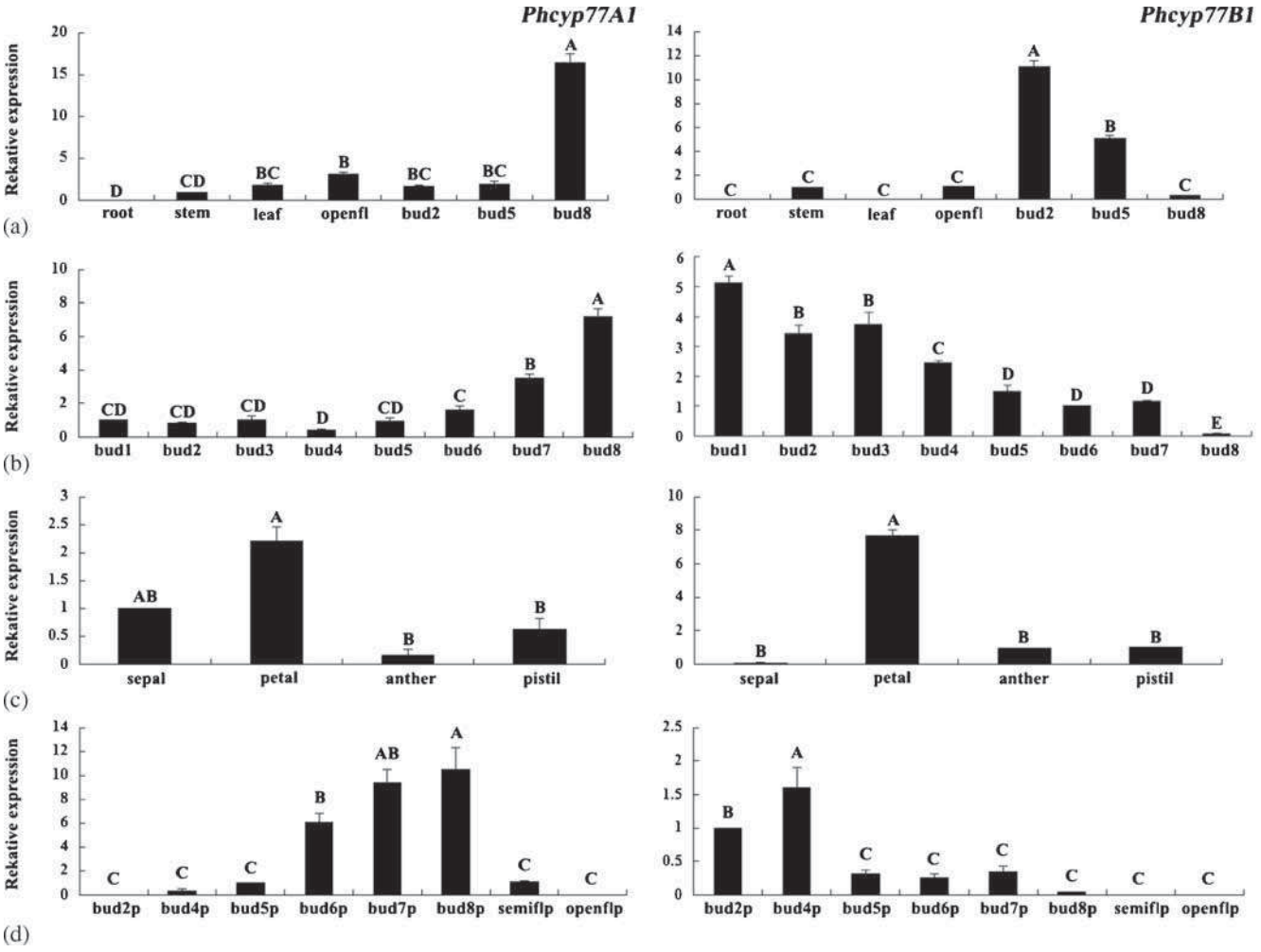

Figure 2. Expression analysis of *Phcyp77A1* and *Phcyp77B1* genes in different plant tissues. (a) *Phcyp77A1* and *Phcyp77B1* genes transcript levels in flower organs and different vegetative organs. (b) *Phcyp77A1* and *Phcyp77B1* genes transcript levels during the flower buds developmental processes. (c) *Phcyp77A1* and *Phcyp77B1* genes transcript levels in the four whorls. (d) *Phcyp77A1* and *Phcyp77B1* genes transcript levels during the petals development processes. Openfl, opening flower; semifl, semi-opening flower; bud2p, bud4p, bud5p, bud6p, bud7p, bud8p, semiflp and openflp mean the petals from bud2, bud4, bud5, bud6, bud7, bud8, semi-opening flowers and opening flowers, respectively. Error bars on each column represented the standard error derived from three replicates. Different capital letters on top of the bars indicate significant differences ($P < 0.01$) by Turkey's multiple range test.

12 and then prominently reduced before flowering (Schmid *et al.* 2005). It has been demonstrated that the *CYP77A6* plays a critical role in the cutin formation before the flower opening and the shape of petals is remarkably changed in *CYP77A6* mutants due to the lack of cuticle and surface nanoridges (Li-Beisson *et al.* 2009). In this research, we find that the transcript level of *PhCYP77A1* is constantly increased with the growth of petals and then significantly decreased during the flower opening stages (figure 2d). The same gene clan and similar gene expression patterns of *Phcyp77A1* and *CYP77A6* lead to the speculation that *Phcyp77A1* could affect the shape of petals by controlling the cutin formation in petunia. In *Arabidopsis*, apart from *CYP77A6*, other CYP77 members also have important functions, such as the *CYP77A4* which could catalyze the *in vitro* formation of antifungal compounds (Sauveplane *et al.* 2009; Bak *et al.* 2011) and the *CYP77A7* which is related to the pollen germination (Boavida *et al.* 2009). The function of *CYP77B1*, which is the only member of CYP77B subfamily, is still unclear. In our study, *Phcyp77B1* is highly expressed in the initial developmental stages of petals and then reduced to undetectable in the petals of half-opening or full-opening flowers (figure 2d). These results reveal that *CYP77B1* might participate in the early stages of petal development in petunia.

In conclusion, we have isolated two CYP77 family members (*Phcyp77A1* and *Phcyp77B1*) from petunia and deduced their affiliation to two subfamilies respectively. *Phcyp77A1* and *Phcyp77B1*, which are highly expressed in the flower buds compared with the vegetative tissues and opening flowers, have different developmental expression patterns in the growing flower buds. Moreover, it is proved that they are predominantly expressed in flower bud petals and their

expression trends are reverse during the petal development. These results suggest that the two genes may play important roles in the initial/late stages of the petal development. This study provides important clues for understanding the function of *CYP77* genes in petunia.

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