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Molecular evolution and functional specialization of chalcone synthase superfamily from *Phalaenopsis* Orchid

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Abstract Plant genomes appear to exploit the process of gene duplication as a primary means of acquiring biochemical and developmental flexibility. The best example is the gene encoding chalcone synthase (CHS, EC2.3.1.74), the first committed step in flavonoid biosynthesis. In this study, we examined the molecular evolution of three CHS family members of *Phalaenopsis* including a novel *chs* gene (*phchs5*), which is slowly evolved. The inferred phylogeny of the *chs* genes of *Phalaenopsis* with other two orchid plants, *Bromoheadia finlaysoniana* and *Dendrobium hybrid*, suggested that gene duplication and divergence have occurred before divergence of these three genera. Relatively quantitative RT-PCR analysis identified expression patterns of these three *chs* genes in different floral tissues at different developmental stages. *Phchs5* was the most abundantly expressed *chs* gene in floral organs and it was specifically transcribed in petal and lip at the stages when anthocyanin accumulated (stage1–4). *Phchs3* and *phchs4* were expressed at much lower levels than *phchs5*. *Phchs3* was expressed in pigmented tissue (including lip, petal and sepal) at middle stages (stages 2–4) and in colorless reproductive tissue at late stage (stage 5). *Phchs4* was only expressed in petal at earlier stages (stage 1–3) and in lip at middle stage (stage 4). These results present new data on differentiation of gene expression among duplicate copies of *chs* genes in *Phalaenopsis*.

Keywords Chalcone synthase · Expression regulation · Gene duplication · *Phalaenopsis* · Phylogenetic

Abbreviations
CHS chalcone synthase
PCR polymerase chain reaction
RT reverse transcriptase

Introduction

Phalaenopsis is an important orchid plant. They come in various colors except blue. Chalcone synthase (CHS, EC2.3.1.74) is the key enzyme in flavonoid biosynthesis pathway which catalyses the condensation of three acetate residues from malonyl-CoA with 4-coumaroyl-CoA to form naringenin chalcone (Heller and Hahlbrock 1980). Flavonoids are the major flower pigments in plants and, hence act as attractants to the pollinators. They also play important roles in many other biological functions, including UV protection (Schmelzer et al. 1988), plant–microbe interaction (Dixon 1986) and male fertility (Taylor and Jorgensen 1992).

Genetic redundancy is a prime feature of plant genomes. Virtually all plant genes so far examined are represented within most plant genomes as small gene families that originate through duplication. Chalcone synthase is encoded by a small family of genes in many plant species, such as *Petunia* (Koes et al. 1989), *Ipomoea* (Durbin et al. 1995), *Gerbera* (Helariutta et al. 1996), and leguminous plants (Ito et al. 1997; Ryder et al. 1987; Wingerder et al.

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1989). The new duplications must be positively selected to persist over long periods of evolutionary time; otherwise the relentless accumulation of mutations would ultimately convert the duplicate copies to a pseudogene. Newly duplicated genes may be of adaptive value to plant in three ways, such are increasing production of product, encoding newly functional protein, or enhancing specialization in developmental expression (Durbin et al. 2000).

In this study, the expression specialization with respect to the *Phalaenopsis* genes that encode chalcone synthase is explored. We presented data on the specialization of three duplicate CHS genes of *Phalaenopsis* in developmental and spatial expression so as to demonstrate the positive selection on gene duplications.

Materials and methods

Plant material

Phalaenopsis hybrida plants were grown under standard conditions (L/D, 16/8 h; at 25–28°C). Samples were collected from green house of Institute of Horticulture, Agricultural Science College of Shanghai, China.

Cloning of phchs5 gene by PCR amplifications

Total RNA of flower (just before open) was extracted using Trizol kit (Sangon Biological Company, Shanghai, China). Revert Aid^{TM}M-MuLV Reverse Transcriptase kit (Fermentas, MBI) was used for the first-strand cDNA synthesis. RT-PCR was performed using two primers: 5′-CCK TCH YTG GAY GCN MGR CAR GAC-3′ and 5′-GG BCC RAA NAR MAC ACC-3′ (Wang et al. 2000). Genomic DNA was extracted and then digested with *Hin*dIII and self-ligated to generate circular molecules. The molecules were used as templates for nested amplifications. According to the sequence of the RT-PCR product, nested primers were designed for inverse PCR: IN1F: 5′-GCC ACT CGG TGT GCT TGA TTG-3′, IN1R: 5′-GTT GAC GGA GGG GCG GAG A-3′; IN2F: 5′-GAG TAG AAA TGT GCT CGC TGA-3′, IN2R: 5′-CGG GTG AGT TGG TAG TCG G-3′; IN3F: 5′-GGC GAG GGT TTG GAG T-3′, IN3R: 5′-TGA GTT ATG CGT GAT TTG G-3′. The following amplification conditions were set: 1 min at 94°C, 30×(20 s at 98°C, 15 min at 65°C) and 10 min at 72°C. First round of PCR was performed with IN1F and IN1R and the product was diluted 50-fold for a second round of amplification with IN2F and IN2R as primers. The third round of amplification was carried out using 50-fold diluted products from second round of PCR as template and IN3F and IN3R as primers. The final PCR product was purified and cloned into T-vector for sequencing.

Sequence comparisons

Sequence comparisons were made between members of CHS superfamily from several species including five *chs* sequences from *Phalaenopsis* for phylogenetic analysis (Table 1). Homology and sequence identity were confirmed using Clustal×ver1.8 (Thompson et al. 1994). The protruding ends of sequences were truncated if necessary. The MEGA2 program (Kumar et al. 2001) was used to estimate the number of nucleotide substitutions per synonymous and nonsynonymous site between groups of sequences (Nei and Gojobori 1986). Neighbor-joining method (Saitou and Nei 1987) was used for phylogenetic analysis. The robustness of the tree tropology was

Table 1 Source of CHS sequences

Taxon	Sequence	Genebank accession no.
Orchid		
Phalaenopsis hybrida	PHCHS3[a]	AY954515
	PHCHS4[a]	AY825502
	PHCHS5[a]	DQ089652
	PHCHS1	U88077
	PHCHS2	AY282575
Bromheadia finlaysoniana	BFCHS1	BFI131830
	BFCHS2	AF007097
Dendrobium hybrid	DHCHS	AY741319
Liliaceae		
Lilium hybrid	LHCHS1	AF169798
	LHCHS2	AF169799
	LHCHS3	AF169800
Araceae		
Anthurium andraeanum	AACHS	AY232492
Gramineae		
Hordeum vulgare	HVCHS1	Y09233
	HVCHS2	X58339
Zea mays	ZMC2	X60205
	ZMWHP1	X60204
Oryza sativa	OSCHS1	X89859
	OSCHS2	XM_477391
	OSCHS3	AB058397
Solanaceae		
Petunia hybrida	PeHCHSA	S80857
	PeHCHSB	X14592
	PeHCHSD	X14593
	PeHCHSF	X14594
	PeHCHSJ	X14597
Convolvulaceae		
Ipomoea purpurea	IPCHSA	P48397
	IPCHSB	P48398
	IPCHSD	O22045
	IPCHSE	O22047

[a] indicates sequences determined in this study

assessed by bootstrap analysis with 1000 resampling replicates.

The method of Muse and Gaut (1994) implemented in RRTree (Robinson-Rechavi and Huchon 2000) was used for relative rate test with *oschs2* as reference sequence. The test was applied to synonymous and nonsynonymous rate separately, with the method of Li (1993) being used for rate estimation.

Expression analysis of chs genes in Phalaenopsis by relative quantitative RT-PCR

Total RNA from various tissues at different developmental stages was isolated using Trizol kit (Sangon Biological Company, Shanghai, China). Revert Aid^{TM}M-MuLV Reverse Transcriptase kit (Fermentas, MBI) was used for the first-strand cDNA synthesis. A pair of primers were designed according sequence of *phchs3* (gene bank accession number: AY954515), RT-F3: 5′-CAG TTA TAG TTG GGG TGG AC-3′ RT-R3: 5′-GCT TCT TTT GCT GAT TTT TT-3′; Primers were designed according to sequence of *phchs4* (gene bank accession number: AY825502), RT-F4: 5′-CGC ATC TGT GAG AAG-3′ and RT-R4: 5′-CCA CCT GGT CAA GAA TCG CT-3′; Primers for *phchs5* amplification: RT-F5: 5′-GCG TGC TCG TCG TTT-3′, RT-R5: 5′-TTG AGT AGG AAG GTG A-3′. The following amplification conditions were used, 3 min at 94°C, 28×(1 min at 94°C, 1 min at 50°C, 2 min at 72°C), 10 min at 72°C. The amounts of *actin* gene transcripts were used as the internal control.

Results

Cloning of phchs5 sequence

The sequence of *phchs5* was obtained by two procedures. Firstly, the exon2 sequence of *phchs5* (about 800 bp) was cloned by RT-PCR amplification. According to the sequence of exon2, inverse PCR was then performed to obtain the other regions of coding sequence and intron sequence. The total sequence of *phchs5* was 1855 bp (gene bank accession number: DQ089652). *Phchs5* showed higher identities (>80% on nucleotide level) with *Bromoheadia chs2* (AF007097), *Ipomoea* CHSD (O22045) and CHSE (O22047), and *Lilium* CHSs (AF169798, AF16799 and AF16800) than with reported *Phalaenopsis chs* genes (U88077, AY282575, AY825502, etc). It is inspiring for us to further investigate the evolution of *chs* genes in *Phalaenopsis*. Cloning of *phchs5* is essential for the following sequence analysis and the research on differential expression regulation of duplicate *Phalaenopsis chs* genes.

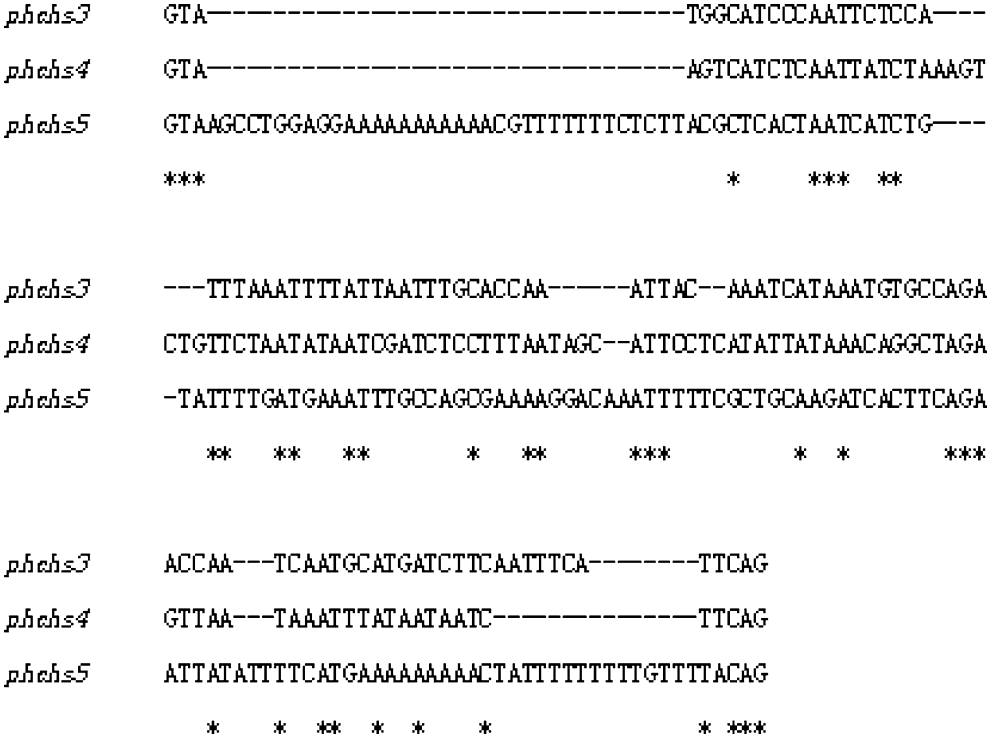

Fig. 1 Alignment of intron sequences of three identified *Phalaenopsis* CHS genes. The stars below each line of alignment indicate conserved sites

Sequence divergence among Phalaenopsis chs genes

Intron sequence comparison among Phalaenopsis chs genes

To date, we have cloned three *chs* genes together with the novel *phchs5* gene identified in this study (GeneBank accession number of the other two *chs* genes are AY954515 (Han et al. 2005) and AY825502 (Han et al. 2006), respectively). Sequence comparison revealed that each gene contained one intron, which interrupted the codon for cysteine at the same location as reported in all chalcone synthase and chalcone synthase like genes (Feinbaum and Ausubel 1988; Sommer and Saedler 1986). The introns are all bordered by 5′-GT and 3′-AG consensus sequences (Brown 1986). The intron sequences of three genes were compared (Fig. 1), which showed that many insertion/deletion events have occurred and their original appearance was completely lost nowadays. The sequence identity between the intron sequences of any two *chs* genes is very low when the *E*-value is set as 10.

Duplication and sequence divergence of Phalaenopsis (Orchid) chs genes

Based on the amino acid sequences, the phylogenetic relationships among Orchid CHSs were inferred by NJ analysis (Fig. 2). It highlighted two important features. First, *Phalaenopsis* CHSs formed two subfamilies. PHCHS1, 2, 3 and 4 clustered together in one branch and PHCHS5 was distributed in another branch. In PHCHS1, 2, 3, 4 families, PHCHS4 was closely related to PHCHS1

Table 2 Absolutely synonymous and nonsynonymous substitution rate (Nei and Gojobori 1986) between *Phalaenopsis hybrid* and the presumed *Bromheadia finlaysoniana* orthologues (*bfchs2* for *phchs5*; *bfchs1* for *phchs1*,*2*,*3* and *4*)

Gene	Synonymous substitutions rate	Nonsynonymous substitution rate
phchs5 to *bfchs2*	0.12589±0.02391	0.15798±0.01375
phchs1 to *bfchs1*	0.13907±0.02290	0.22625±0.01807
phchs2 to *bfchs1*	0.13867±0.02283	0.22675±0.01807
phchs3 to *bfchs1*	0.11190±0.02062	0.24675±0.01807
phchs4 to *bfchs1*	0.13942±0.02190	0.23036±0.01831

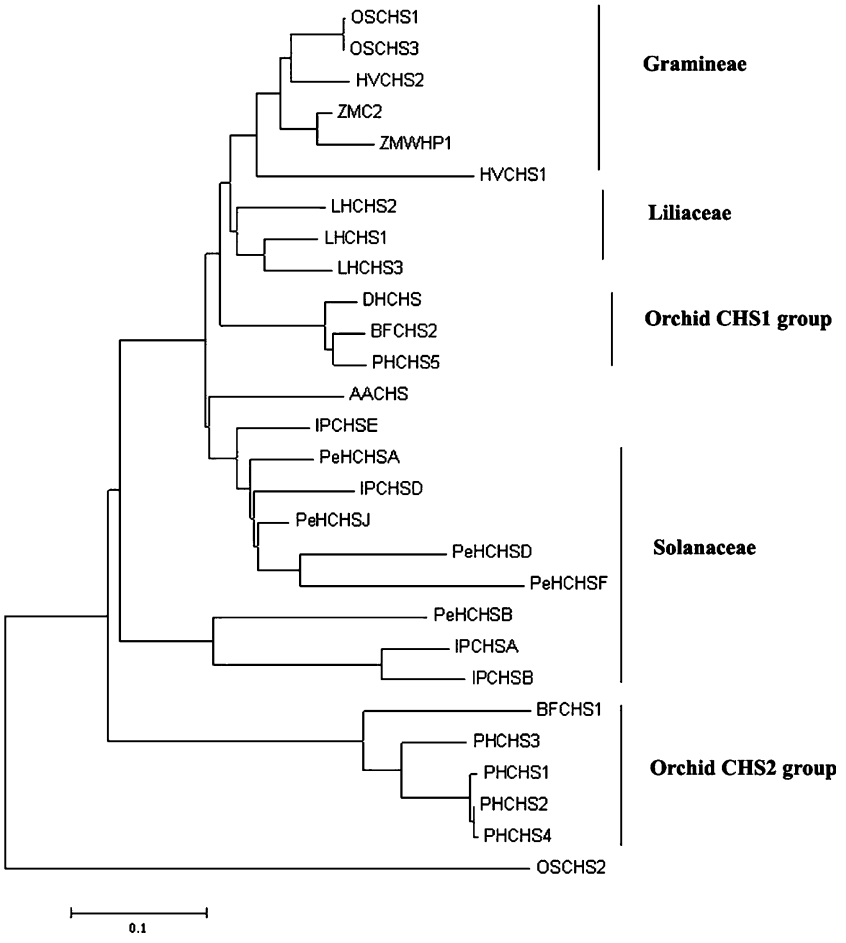

Fig. 2 Neighbor joining tree for *chs* genes from *Phalaenopsis* and other genera of orchid family. Sequences are identified both by species and the clone number. See Table 1 for abbreviations. The bootstrap is 1000

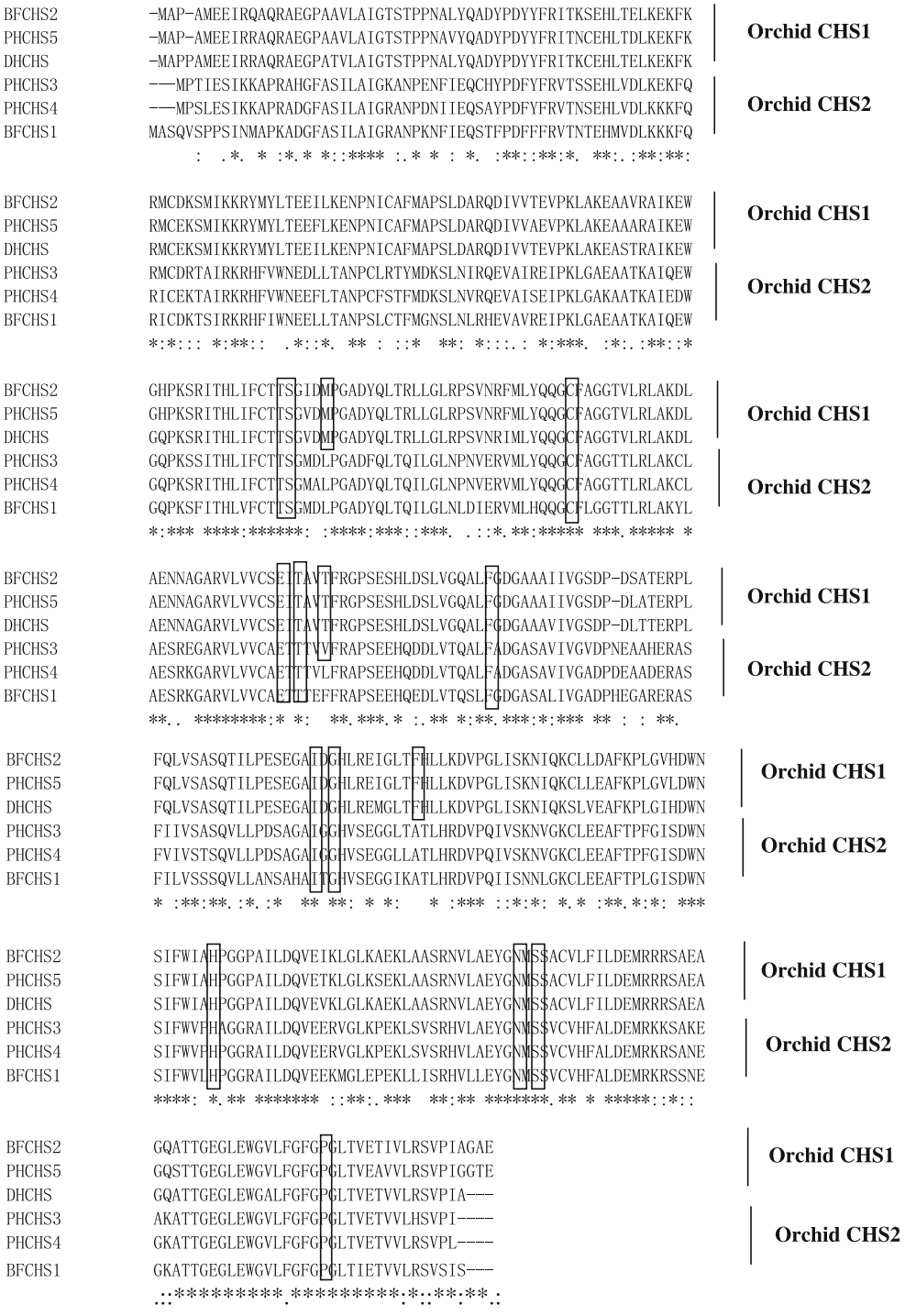

Fig. 3 Alignment of the amino acid sequences of CHSs from orchid family. The conserved residues were framed (Ferrer et al. 1999). They were T132, S133, C164, I254, G256, H303, N336, S338, P375. The active residues M137, T197 and F265 were absent from sequences of Orchid CHS2 group. The stars below each line of alignment indicate conserved sites

and PHCHS2, both of which are cloned by other researchers. Secondly, Orhcid CHS (including *Phalaenopsis hybrid*, *Bromoheadia finlaysoniana* and *Dendrobium hybrid*) did not form genus-specific clusters. Instead, all the examined Orchid CHS fell into two subfamilies. PHCHS5 was more closely related to *Bromoheadia* CHS2 and *Dendrobium* CHS (designated as Orchid CHS1 group). However, PHCHS1, 2, 3 and 4 clustered with *Bromoheadia* CHS1 (designated as Orhcid CHS2 group) (Fig. 2). The phylogenetic analysis thus suggests that

Orhcid *chs* genes have diverged into two subfamilies before the divergence of these genera. According to Fig. 2, orchid CHS2 group have long-branch length within the phylogeny, suggesting accelerated rates of nucleotide substitution. However, Orchid CHS1 group have shorter branch length and slower rates of nucleotide substitution.

Absolute rates of synonymous and nonsynonymous substitution were calculated between *Phalaenopsis chs* genes and the presumed *Bromoheadia* orthologues (Table 2). The results showed that synonymous rates were homogeneous for phchs1,2,3,4 and 5. At the nonsynonymous sites, the calculation revealed that *phchs1*,*2*,*3*,*4* subfamily is evolving one and a half times faster than *phchs5* after the divergence of these two genera.

Alignment of the amino acid sequences of the orchid CHSs revealed that these CHSs contained most of the active sites including T132, C164, E192, T194, F215, H303, N336, P375 (Ferrer et al. 1999). But several active amino acids including F265, M137, T197 were absent from protein sequences of Orchs2 group (Fig. 3). T197 constitutes the binding pocket for coumaroyl-CoA with other amino acid residues. F265 and M137 are part of pocket, which serves for cyclization reaction (Ferrer et al. 1999).

Molecular clocks and evolutionary rate of Orchid CHS groups

To examine the rate variation between the two lineages of the *chs* genes in orhcid family, the relative rate test of Muse and Gaut was performed (1994). Codon models were used to test whether the two lineages followed either a nonsynonymous or a synonymous clock. The outgroup sequence in all comparison was *chs2* of *Oryza sativa* (*oschs2*). Rate tests show that neither of the lineages differs significantly in rates of synonymous substitution, although at distant levels of comparison this result may be confounded by saturation of synonymous positions. The rate of nonsynonymous substitution is, however, significantly faster in genes of Orchid CHS2 group than the more conserved Orchid CHS1 group genes with $P < 0.0001$, df=1. The nonsynymous rates do not differ significantly among *phchs5*, *dhchs* and *bfchs2*, or among *phchs1*,*2*,*3*,*4* and *bfchs1*.

As the molecular clock assumption appears to hold at synonymous sites, synonymous rates was used to calculate rough estimates of the gene duplication times. The absolute rate of synonymous substitutions per site between Orchid CHS1 group and Orchid CHS2 group was 0.3171. The average synonymous substitution rate for plant nuclear genes was about 5×10^{-9} substitutions per site per year (Li 1997, p 193). Thus the times of duplications was estimated to be more than 31 million years ago.

Expression divergence among Phalaenopsis chs genes in floral organs

Figure 4 shows the expression of different CHS genes in various tissues and stages of development in *Phalaenopsis* as determined by relative quantitative RT-PCR using gene specific primers. Development of *Phalaenopsis* flower was divided into five stages (Fig. 5). Tissue was dissected from flower bud (in mauve color) at stages 3, 4 and 5.

It is evident from RT-PCR results that the timing and tissue specificity of expression of the various *chs* genes in *Phalaenopsis* are quite variable, suggesting that there is considerable divergence in the developmental regulation of individual members of the gene family. RT-PCR revealed that none of these three *chs* gene was transcribed in leaves and roots of intact plant growth and developmental stage (data not shown).

Phchs5 was by far the most abundantly expressed *chs* gene in petals. It was only expressed in petal and lip tissue (in *Phalaenopsis*, lip is transformed petal). The peak level of *phchs5* mRNA expression was in petal tissue at stages 3–4 when the anthocyanin was accumulated at the highest rate. *Phchs5* stopped to be transcribed at stage 5 in petal when the flower was fully open. *Phchs4* was another *chs* gene, which was specially expressed in petals and lips. It was expressed at a low level compared with *phchs5*. *Phchs4* was weakly transcribed in petal of stage 3 and so was *phchs3*, another weakly expressed *chs* gene in floral organs. In the lip of flower, *phchs3* was expressed at higher level than either *phchs4* or *phchs5*. It was moderately transcribed in lip of stage 3 while *phchs5* was expressed weakly at the same stage. The transcripts of *phchs4* could be weakly detected in lip at stage 4.

Phchs3 was moderately expressed in sepals at stage 4 and weakly expressed in column at stage 5. It was the sole *chs* gene, which was expressed in sepal and column in this study. *Phchs3* was the sole *chs* gene expressed in reproductive organs and it may encode CHS protein, which is responsible for fertility.

As far as the developmental stage was concerned, the peak of expression of *phchs3* in floral organs occurred earlier than *phchs5*. The highest expression of *phchs3* occurred at stage 2 while *phchs5* was expressed at the highest at stages 3 and 4. The expression of *phchs5* apparently corresponds to the pigmentation of petals and it may be responsible for anthocyanin biosynthesis in petals. Considering the low expression level of *phchs3* and *phchs4* in floral tissue, these two genes may not be the major *chs* gene for anthocyanin biosynthesis.

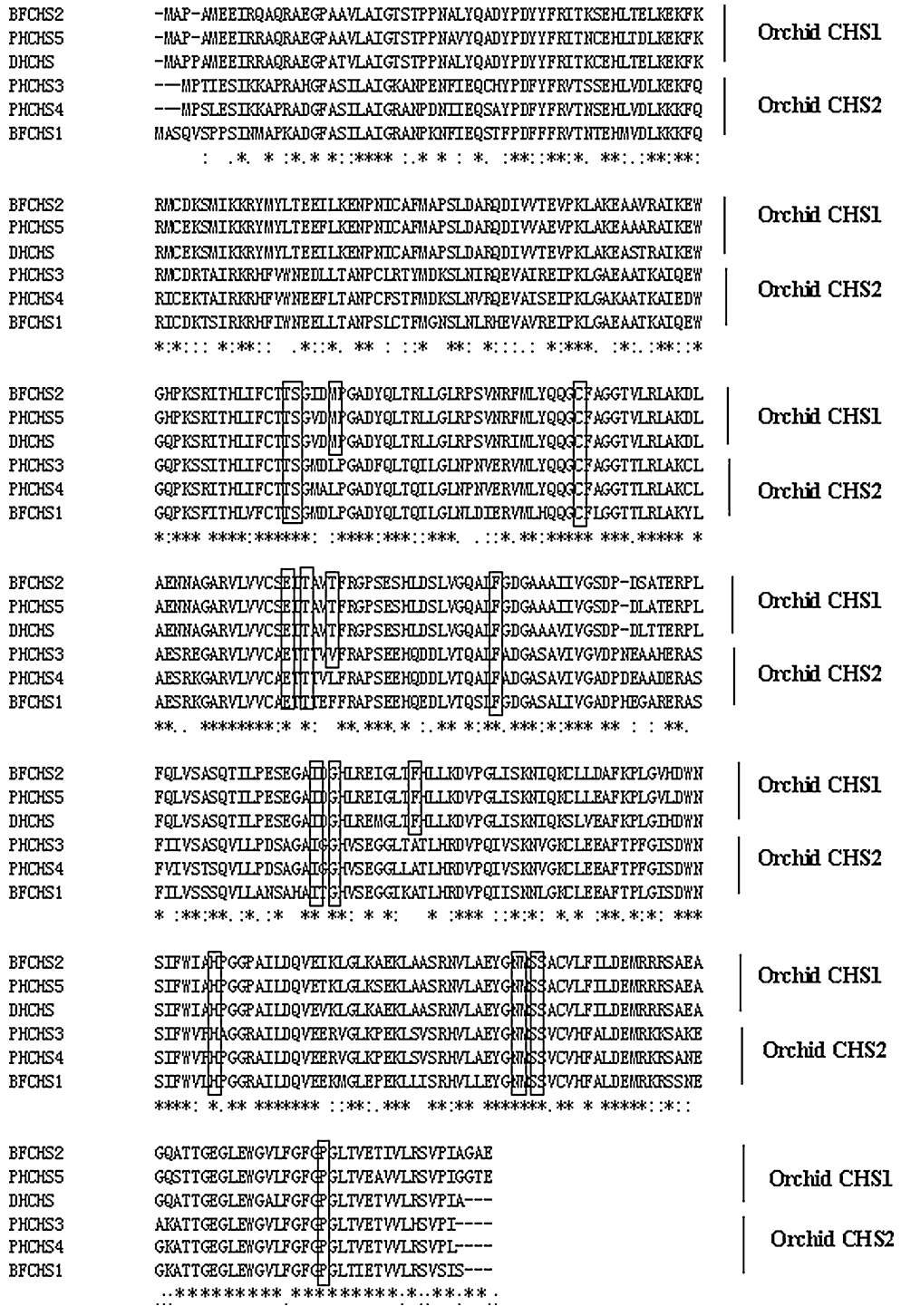

Fig. 4 Expression of different *chs* genes in various organs and stages of development in *Phalaenopsis hybrid* (in mauve color) by RT-PCR. The amounts of *actin* transcripts were used as internal control. Primers used for the amplification are positioned at the non-conservative regions

Expression of phchs5 in different Phalaenopsis cultivars

To investigate the correlation between expression of *phchs5* and the pigmentation of flower, we performed RT-PCR (Fig. 6) using the samples from three *Phalaenopsis* cultivars that show different flower color type (Fig. 7). *Yel* cultivar has yellow sepal and petal and mauve-dotted lip; *Wht* has white sepal and petal and yellow lip; and *Mau* has mauve sepal and petal and purple lip. The column is white in all the cultivars. Data revealed that *phchs5* was not expressed in sepal and

Fig. 5 Developmental stages of the flower in *Phalaenopsis hybrida*. Stage 1 is ca. 0.5 cm and the flower organs slightly pigmented. Stage 2 is ca.1.0 cm and the anthocyanin goes on being accumulated. Stage 3 is ca. 2 cm. In this stage, anthocyanin is accumulated at fast rate. In stage4, the flower is just open and the anthocyanin accumulation comes to the highest level. In stage 5, the flower is fully open and each parts of perianth are fully expanded and the pollen is dehiscing. At this stage, anthocyanin biosynthesis is expected to be fulfilled or have been finished yet. Flower tepal includes lip, sepal and petal

column at any developmental stages in *Yel* and *Wht* like in *Mau* (data not shown). In *Yel*, *phchs5* was expressed in similarly tissue-specific way with *Mau*, i.e. it was transcribed in petal and lip, but the expression level was much lower than in *Mau*. *Phchs5* was not expressed until stage 3 in *Yel* while was transcribed from stage 1 in *Mau*. Maybe it results from the fact that floral organ of *Yel* is wholly white at stages 1 and 2, and the petal, sepal and lip begin to be pigmented from stage 3. Although petal is wholly white in *wht*, *phchs5* was moderately transcribed in petal of stage 4. This color phenotype probably is caused by the mutations in other downstream genes. Maybe the lower expression level of *phchs5* is caused by feedback inhibition of some flavonoids. Above data indicated that *phchs5* was specially expressed in petal tissue and its expression level is apparently consistent with anthocyanin accumulation in this organ. Although lip of *Wht* is in yellow color, *phchs5* was not expressed in this tissue as in other cultivars. Probably its expression in lip is dependent on the pigmented degree of this tissue. If the lip is pigmented lightly (for example, in yellow color), *phchs5* is not expressed (Fig. 6, line of L*wht*). If the lip is pigmented in darker color (for example, in mauve or purple color), *phchs5* is expressed weakly (Fig. 6, lines of L*yel* and L*mau*). Although sepal and petal are in identical color as far as each cultivar is concerned, *phchs5* was not transcribed in sepal in any cultivar.

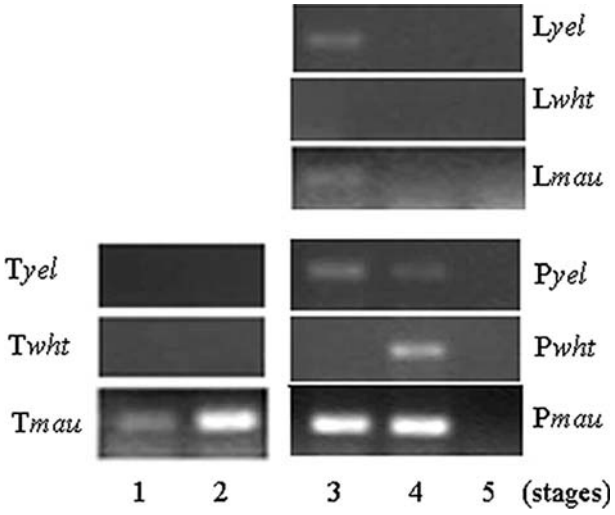

Fig. 6 Expression of *pchs5* in lip and petal tissue in three cultivars of different color phynotype (*yel*, *wht* and *mau*). The division of developmental stage is similar with that of Fig. 4. Relatively equal amounts of first strand cDNA are used in each reaction. This is determined by amounts of *actin* transcripts. L, lip; T, tepal; P, petal

Discussion

In this study, we successfully got to several achievements in exploring molecular evolution of *Phalaenopsis* and Orchid *chs* genes as well as expression regulation of *Phalaenopsis chs* genes.

Fig. 7 Phenotype of three different *Phalaenopsis* cultivars

One novel *Phalaenopsis chs* gene was obtained, which is far diverged from *Phalaenopsis* CHSs previously published and showed higher identities with CHS of other orchid plants. It extends the knowledge about *Phalaenopsis* and plant CHS genes and will contribute much to the research on the molecular evolution of this gene family.

Based on the cloning and sequencing of this novel gene, we obtain the information that *chs* genes of orchid plants have been diverged into two subfamilies before the divergence of genera including *Phalaenopsis*, *Bromheadia* and *Dendrobium*. One branch (Orchid CHS1 group) evolved slowly and appears to be under greater selective constraints. Genes of this branch may be responsible for anthocyanin biosynthesis. The other branch is rapidly evolving with higher rate of accumulation of amino acid change. It is unclear whether this is also the way in other genera of orchid family.

However, this evolutionary split is also mirrored in such plant species as *Petunia*, *Ipomoea* (Durbin et al. 2000), and *Asteraceae* plant family including *Dendranthema* (Yang et al. 2002) and *Gerbera* (Helariutta et al. 1995). Probably this is a general law in evolution of botanic *chs* genes. What should be further investigated is whether it is a common phenomenon that divergence of CHS occurred before divergence of all the genera of one plant family.

Sequence divergence is often in conjunction with divergence in expression regulation. In *Ipomoea*, expression of the slowly evolved CHSD gene is consistent with the pigmentation of flower limb, while there is no distinct correlation between flower pigmentation and expression of the rapidly evolved CHSA, B, C genes (Habu et al. 1998; Durbin et al. 2000). In *Petunia*, the distantly evolved CHSB is not expressed in floral organs but can be induced by UV irradiation (Koes et al. 1989). In this study, expression analysis by RT-PCR exhibited that there has been shifts in developmental expression among CHS gene family members in *Phalaenopsis*. RT-PCR showed that *phchs5* is expressed at the highest level in floral organs among these three examined genes, which apparently corresponds to the appearance of pigments. *Phchs5* is evidently the sole *chs* gene responsible for the accumulation of pigment in petal. *Phchs3* and *phchs4* were expressed at much lower level than *phchs5* in floral organs and they are not the major *chs* genes for anthocyanin biosynthesis according their low transcription level (Durbin et al. 2000).

Sequence divergence is also usually in conjunction with shifts in enzyme function (Axelsen and Palmgren 1998). Biochemical analysis of *Ipomoea* CHS A, B, D and E revealed that only CHSD and E are capable of catalyzing the condensation reaction that results in naringenin chalcone (Shiokawa et al. 2000). Detailed sequence comparison among Orchid CHSs showed that several active sites (M137, T197 and F265) that are thought to be important in enzyme specificity were absent in genes of Orchid CHS2 group. These differences may lead to the differences in substrate specificity and reaction patterns between genes of these two groups. *Phchs4* showed high identity (about 98% on nucleotide level) with bibenzyl synthase (Preisig-Müeller et al. 1995), a gene involved in microbe–plant interactions. It can be inferred that genes of orchid CHS2 group may be mainly responsible for defense reaction in plants.

It is unclear why there has been no *chs* genes abundantly expressed in sepals as in petals although sepal is in same color with petal. There are two possibilities that can explain this kind of phenomenon. First, another *chs* gene that is far diverged from known *chs* genes is responsible for its pigmentation. If this is the truth, CHS might have diverged into three subfamilies in orchid plants. Second, considering that sepal and petal are uniformly pigmented as for each *Phalaenopsis* cultivar, there may exist an anthocyanin transport system that can transport anthocyanin from petal to sepal.

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