Cloning of the segment of flavonoid 3′-hydroxylase gene from the gDNA of *Prunus mume* by degenerate PCR

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Abstract: The gDNA was extracted from the tender leaves of *P. mume* with "Predeimpurity-SDS method" developed in this study. Based on the highly conserved amino acid regions of the 11 deduced amino acid sequences of the cDNAs of the flavonoid 3′-hydroxylase (*F3′H*) genes which had been openly published and submitted in the GenBank databases, 2 forward degenerate primers and 3 reverse degenerate primers were designed and consisted of 6 sets of primer pair. Only one primer pair could be used in degenerate PCR to amplify three 469 bp segments from the gDNAs of *P. mume* 'Nanjing Hongxu', *P. mume* 'Nanjing Hong' and *P. mume* 'Fenpi Gongfen' respectively. Sequence analysis indicated that the 3 segments shared 99.72% identity among each other and 65.57% identity with the corresponding regions of the 11 cDNAs as a whole. Furthermore, the "GGEK" motif is pointed out not to be the characteristic sequence of F3′H. It is the first time for the segment of *F3′H* gene to be cloned from the gDNA of ligneous plant. This study can underlay the cloning of the full length of *F3′H* gene from *P. mume*.

Key words: *P. mume* Sieb. et Zucc.; extraction of gDNA; segment of flavonoid 3′-hydroxylase gene; degenerate PCR

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Mei (*Prunus mume* Sieb. et Zucc.) flower is one of the candidates of the national flower of P. R. China today. The scientific research of sixty years on Mei flower in China has obtained superexcellent achievements. However, the scientific study on the flower color of Mei is almost a blank (Chen, 2002; Li, 2004).

The flower color of Mei includes mauve, pink, pure white, greenish white, light yellow and double color (Chen, 1989). *P. mume* 'Nanjing Hongxu', belonging to Form Cinnabar, is the typical representative of the mauve. *P. mume* 'Nanjing Hong' and *P. mume* 'Fenpi Gongfen', all belonging to Form Pink Double, are the typical representatives of the pink (Chen, 1989). The anthocyanins of the flower color pigments of these 3 cultivars have been identified as cyanins (Zhao *et al.*, 2004a, b, 2006), indicating that the key enzyme determining the biosynthesis of the anthocyanins of the flower color pigment of Mei is flavonoid 3′-hydroxylase (F3′H).

F3′H is a microsomal cytochrome P450-dependent monooxygenase that requires NADPH as a co-factor (Forkmann, 1991), which was first dem-

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onstrated in microsomal preparations from cultured *Haplopappus gracilis* cells(Fritsch *et al.*, 1975). It controls the hydroxylation of dihydrokaempferol to dihydroquercetin and of naringenin to eriodictyol (Brugliera *et al.*, 1999). As a result, F3′H determines the shift from pelargonidin to cyanidin(Tanaka *et al.*, 1998)and results in the accumulation of cyanidin derivatives, which leads to red flower color.

As membrane-bound and P450-dependent protein, F3′H is difficult to isolate. Furthermore, cloning and identification of flavonoid 3′-hydroxylase gene(*F3′H*)by homology to the highly conserved regions shared by the P450 family in plants is complicated by the large number of P450-like sequences(Schuler, 1996; Chapple, 1998). Till 1999, isolation of a *F3′H* cDNA clone from *Petunia hybrida* was first reported(Brugliera *et al.*, 1999).

Degenerate polymerase chain reaction(PCR), namely degenerate oligonucleotide-primed PCR (DOP-PCR)(Telenius *et al.*, 1992), uses degenerate primers to accomplish PCR. Degenerate primers are a set of primers which have a number of options at several positions in the template sequence so as to allow annealing to and amplification of a variety of related sequences(Innis *et al.*, 1990). They are radically resulted from the degeneracy of the genetic code because primers targeted to particular amino acid sequences must be degenerate to encode the possible permutations in that sequence. The obvious advantage of degenerate PCR is that the nucleotide sequences of the target genes are obtained by amplifying according to the conserved amino acid sequences of the target genes' homologous protein. As a result, it has been widely applied in the cloning of new gene, the analysis of gene expression and so on(Shi *et al.*, 2004).

We report in this paper the amplification of the segment of *F3′H* from the genomic DNA(gDNA)of *P. mume* by degenerate PCR with the objective to underlay the starting point for cloning the complete sequence of the *F3′H*. It is the first time for the segment of *F3′H* to be cloned from the gDNA of ligneous plant.

1 Materials and methods

1.1 Materials

1.1.1 Plant materials All leaves of *P. mume* 'Nanjing Hongxu', *P. mume* 'Nanjing Hong' and *P. mume* 'Fenpi Gongfen' were obtained in the Research Centre of Mei flower of Sun Yat-sen Mausoleum Administrative Office of Nanjing. Tender leaves were collected randomly, encased in white gauze pocket, immediately frozen in liquid nitrogen. Then the leaves were quickly ground into powder in a white porcelain pestle after adding approximate 15 mL liquid nitrogen and frozen at -80℃ until the extraction of gDNA.

1.1.2 Plasmid and strain DNA cloning pMD18-T vector kit was the product of TaKaRa Biotechnology (Dalian) Co., Ltd. *Escherichia coli* (*E. coli*) DH5α was conserved in our laboratory.

1.1.3 Enzyme and reagents *Thermus aquaticus* (*Taq*) DNA polymerase, namely *TaKaRa Taq*™, and TaKaRa Agarose Gel DNA Purification Kit Ver. 2.0 were the products of TaKaRa Biotechnology(Dalian)Co., Ltd. *Eco*RⅠ, Sodium Dodecyl Sulphate(SDS), β-mercaptoethanol(BME)and tetramethyl ammonium chloride(TMAC)were the products of Amresco. RNase A and agarose were the products of Sigma. Distilled deionized water (ddH_2O) was used for all buffers and reagents. Ampicillin (Amp), Isopropyl-β-D-thiogalactoside (IPTG), tris-acetic acid-EDTA buffer(TAE)and tris-boracic acid-EDTA buffer(TE)(pH8.0))were filtered though 0.2 μm membrane. 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) was dissolved in N, N-dimethylformamide at 20 mg/mL, and stored at -20℃ in a foil wrapped tube.

Other reagents were of analytical grade made in China.

1.2 Methods

1.2.1 General All plastic supplies, including microfuge tubes and pipet tips, were sterilized by autoclaving. Centrifugation was performed in 3K30 centrifuge(Sigma). The absorption values of DNA

were determined at room temperature(RT) in a 1 cm pathlength quartz cell using a Ultrospec R 3000 UV-Vis spectrophotometer(Amersham Pharmacia Biotech).

Agarose gel electrophoresis was carried out in horizontal electrophoretic trough using 1 × TAE (pH8.0) as electrophoresis buffer. Ethidium bromide(EB) was directly mixed into the gel. 6× loading buffer was composed of 30 mmol/L EDTA, 36% glycerol, 0.05% xylene cyanol FF and 0.05% bromophenol blue. The voltage was 5 V/cm.

Thermal cycling of degenerate PCR was performed in the PTC-200 Peltier Themal Cycler(M J Research, Watertown, MA, USA).

1.2.2 Extraction of gDNA from leaf The gDNAs of the three cultivars were isolated from the tender leaf with"Predeimpurity-SDS method"developed in this study.

5 g leaf powder was ground to be mushy after mixing with 5 mL"deimpurity buffer(0.4 mol/L glucose, 3% polyvinylpyrrolidone(PVP)(Couch *et al.*, 1990; Kim *et al.*, 1997; Fu *et al.*, 1998), 0.3% β-mercaptoethanol (BME))", and centrifuged at 10 000 g for 10 min at 4℃. The supernatant was discarded.

After being treated again with the same buffer and same method, the precipitate was added 10 mL preheated at 65℃"extraction buffer(100 mmol/L Tris-HCl pH8.0, 500 mmol/L NaCl, 50 mmol/L EDTA pH8.0, 1.5% SDS, 0.3% BME)(Dellaporta *et al.*, 1983)", mixed reversedly and gently and heated in 65℃ water bath for 1 h while being shaken gently now and then. The mixture was cooled to RT, added 5 mL of 5 mol/L KCl, mixed reversedly and gently again, then ice-bathed for 20 min. The icy mixture was centrifuged at 7 000 g for 20 min at 4℃ and the corresponding supernatant was taken out and added equivalent volume of chloroform-iso-pentanol (24:1, v/v) and reversed slowly for several times to make it adequate. The solution was centrifuged at 12 000 g for 10 min at 4℃, the supernatant(water phase) was added 0.6 volume of iso-propanol which was frozen in -20℃ in advance, reversed slowly for several times and placed quietly at RT for 1 h. The white floc was drawn out with a glassy hook and blotted up on germfree filter paper. After being washed with 70% ethanol for three times, each for 10 min, the floc was dried at RT and dissolved in 500 μL TE, then added 10 μL RNase A and placed in 37℃ water bath for 1h. The solution was added equivalent volume of chloroform- iso-pentanol(24:1, v/v), reversed slowly for several times and centrifuged at 12 000 g for 15 min at 4℃. The supernatant(water phase) was added 0.1 volume of 3 mol/L NaAc and 2 volume of absolute ethanol which was frozen in -20℃ in advance, placed at RT for 30 min and centrifuged at 7 000 g for 15 min at 4℃. The corresponding precipitate was washed with 70% ethanol for three times, each for 10 min, then dissolved in 500 μL Tris-EDTA buffer and stored at -20℃.

The quality and concentration of gDNA extracted were determined by spectrophotometry, digestion of restriction endonuclease (*Eco*R Ⅰ) and 1% agarose gel electrophoresis analyses.

1.2.3 Design of degenerate primers Degenerate primers were designed on the basis of the multiple alignment of the deduced amino acid sequences of the cDNAs of the *F3′Hs* which had been openly published and submitted into the GenBank of National Centre for Biotechnology Information(NCBI, America). Multiple alignment was constructed with DNassist 2.2.

1.2.4 Amplification of the segment of the *F3′H* by degenerate PCR The degenerate PCR reactions were conducted in 200 μL thin-walled micro-centrifuge tubes and performed in 50 μL reaction volumes(Table 1). In degenerate PCR, TMAC has been used to increase the yield and specificity of PCR products because it is thought to alter the kinetics reactions of primers and templates so as to increase the frequency of the specific annealing between primers and templates(Chevet *et al.*, 1995).

Hot start PCR was as follows: Apart from *TaKaRa Taq*™, all reaction components were confected on ice and mixed equably. Soon after being

heated at 96℃ for 5 min, the system was immediately cooled to 0℃ in icy water bath, then added *TaKaRa Taq*™. The subsequent programs of PCR were as follows: 35 cycles of 95℃ for 1 min, 47～51℃ (The concrete annealing temperature was set according to the special melting temperatures (Tms) of the primer pairs, and was about 5℃ below the lowest Tm of the primer pair to be used (Innis *et al.*, 1990)) for 2 min and 72℃ for 2 min, followed by a 7 min extension at 72℃.

Table 1　Reaction system of degenerate PCR

Reaction components	Stock solution concentration	Working solution preparation (μL)	Final concentration
ddH_2O		28.5	
PCR buffer(Mg^{2+} free)	10×[a]	5.0	1×[b]
$MgCl_2$	25 mmol/L	6.0	3.0 mmol/L
dNTP	10 mmol/L	1.0	0.2 mmol/L
Forward primer	15 μmol/L	1.5	0.45 μmol/L
Reverse primer	15 μmol/L	1.5	0.45 μmol/L
Template	1.0 μg/μL	5.0	100 ng/μL
TMAC	50.0 μmol/L	1.0	1.0 μmol/L
Taq DNA polymerase	5 U/μL	0.5	0.05 U/μL
Total volume(μL)		50.0	

Note: [a]PCR buffer (Mg^{2+} free) (10×): 100 mmol/L Tris-HCl pH8.3 (25℃), 500 mmol/L KCl. [b]PCR buffer (Mg^{2+} free) (1×): 10 mmol/L Tris-HCl pH8.3 (25℃), 50 mmol/L KCl.

1.2.5 Inspection, purification and cloning of the degenerate PCR product The PCR products were analyzed by 2% agarose gel electrophoresis. The band was excised from the gel and purified with the TaKaRa Agarose Gel DNA Purification Kit, then ligated into pMD18-T Vector according to the manufacturer's recommendations.

E. coli DH5α competent cells were prepared by $CaCl_2$ method in advance (Sambrook *et al.*, 2002a).

10 μL recombinant plasmid was transformed into 100 μL the competent cells using heat-shock method, added 900 μL liquid luria-bertani (LB) medium (Amp free) and cultured at 150 rpm for 1 h at 37℃. 100 μL bateria solution was mixed with 40 μL X-Gal and 4 μL of 200 mg/mL IPTG, inoculated on LB-Amp plate (Ø9.0 cm) and stored reversedly at 37℃ for 12～16 h.

1.2.6 Selection and identification of positive clones The white colony was randomly selected and inoculated in liquid LB medium which contained 100 μg/mL Amp, and cultured at 200 rpm for 6 h at 37℃. 5 μL of the subsequent bacteria solution was used as PCR template solution to identify the positive clone (Sambrook *et al.*, 2002b), and the corresponding primer pair was used as the PCR primers. The PCR program was as follows: 95℃ for 5 min, followed by 35 cycles of 95℃ for 1 min, 47～51℃ for 2 min and 72℃ for 2 min, followed by a final extension of 72℃ for 7 min. During the denaturation of 5 min, the *E. coli* cells rived and released the recombinant plasmid (Sambrook *et al.*, 2002b).

1.2.7 DNA sequencing and analysis The positive clone solution was added 50% glycerol till the final concentration of glycerol was 15%, and subjected to sequencing by Invitrogen Biotechnology Co. Ltd. Multiple alignments of the 3 segments of the *F3′H*s amplified from the 3 cultivars of *P. mume* and of the 3 segments with the corresponding regions of the 11 cDNAs were constructed with DNAMAN 4.0.

2　Results and analyses

2.1 Extraction of the gDNA from the tender leaves

The gDNAs extracted from the tender leaves of the 3 cultivars of *P. mume* are all purely white solid or transparent gelatinoid. Their A_{260}/A_{280} s range from 1.7 to 1.9 (Table 2), indicating that the biomacromolecule impurities such as proteins and polysaccharids have been eliminated to a great extent. The A_{260}/A_{230} s of the gDNAs are all bigger than 2.0 (Table 2), indicating that the small molecules such as phenols and amino acids have also been wiped off (Clark, 1998). So the gDNAs have basically met the demands of downstream manipulations.

1% agarose gel electrophoresis analysis revealed the high quality of the gDNAs extracted (Fig. 1: A). The clear, none-dispersive and none-tailing bands showed the gDNAs are comparatively

unabridged. Comparing with the DNA marker, the gDNAs are found to be longer than 50 kb. There is not any fluorescence before the band of bromophenol blue, denoting that no RNA leftover exists in the gDNAs. Moreover, no fluorescence was observed in the holes of sample-adding, implying no viscous impurities, e. g. polysaccharide, remains in the gDNAs. It was also showed by electrophoresis that the gDNAs can be completely digested by *Eco*R Ⅰ (Fig. 1;B).

Table 2 Appearance and ultraviolet absorption characteristics of the gDNAs extracted from the tender leaves of 3 cultivars of *P. mume*

Cultivar	Appearance	Ultraviolet absorption characteristics	
		A_{260}/A_{280}[a]	A_{260}/A_{230}[a]
P. mume 'Nanjing Hongxu'	transparent gelatinoid	1.725	2.088
P. mume 'Nanjing Hong'	transparent gelatinoid	1.738	2.104
P. mume 'Fenpi Gongfen'	purely white solid	1.809	2.066

Note: [a] A_{260}/A_{280} and A_{260}/A_{230} are the average values of four experiments respectively.

2.2 Design of the degenerate primers based on multiple alignment

11 *F3′Hs*, including those of *Arabidopsis thaliana* ecotype="Columbia", *Arabidopsis thaliana* ecotype="Landsberg erecta", *Glycine max* 'Williams', *Glycine max* 'To7B', *Ipomoea purpurea* (common morning-glory), *I. nil* Magen, (Japanese morning glory), *I. tricolor* 'Heavenly Blue', *I. quamoclit*, *Petunia hybrida*; *Torenia hybrida*; *Oryza sativa* (japonica cultivar-group), had been openly published and submitted into the GenBank of NCBI. It was a great pity that all of these *F3′Hs*' cDNAs were obtained only from herbaceous plants, no DNA sequence of *F3′H* was obtained and no *F3′H* was cloned from Rosaceae.

By aligning the 11 complete deduced amino acid sequences of the cDNAs of the *F3′Hs*, four most highly conserved amino acid regions were observed and converted into DNA sequence, from which degenerate primers, namely two forward primers (FPs) and three reverse primers (RPs), were designed (Fig. 2, Table 3). What needs to be explained is that two of the four conserved amino acid regions are thought to be important to the activity of F3′H. The "P" of "LPPGP" is the conserved proline-rich residues that are important for P450 topology and "LPPGP" is thought to act as a hinge that is important for the optimal orientation and targeting of the enzyme to the microsomal membrane (Murakami *et al.*, 1994). The "IPFGAGRRIC" is the signature sequence for the heme-binding domain (FxxGxxxCxG) of P450 enzymes and the "C" of the "IPFGAGRRIC" s the conserved heme-binding cysteine residue serving as the fifth ligand to heme iron (Chapple, 1998; Lewis *et al.*, 1998). In the designed primers, the use of inosines for substituting 4 base wobbles instead of using all 4 base substitutions can lower the degeneracy of primers, resulting in the increase of the concentration of a specific primer and the decrease of the background noise (Innis *et al.*, 1990).

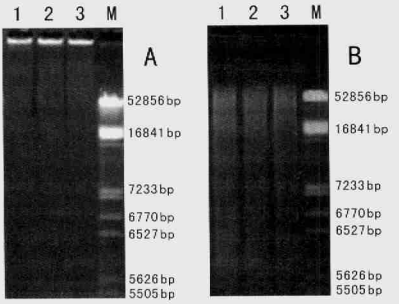

Fig. 1 1% agarose gel electrophoretogram of gDNAs extracted from the tender leaves of the 3 cultivars of *P. mume*

A. Intact gDNAs; **B.** *Eco*R Ⅰ-digested gDNAs. **Lane 1**: *P. mume* 'Nanjing Hongxu'; **Lane 2**: *P. mume* 'Nanjing Hong'; **Lane 3**: *P. mume* 'Fenpi Gongfen'; **Lane M**: DNA marker. The concrete sizes of the bands produced by DNA marker were shown on the right.

It was reported by Brugliera *et al.* (1999) and Zabala *et al.* (2003) that "GGEK" motif was the characteristic sequence of F3′H by which F3′H and flavonoid 3′,5′-hydroxylase (F3′,5′H) were distinguished. But in this study, multiple alignment of

the 11 deduced amino acid sequences of the cDNAs of the *F3′Hs* showed, for the first time, the "GGEK" was not the characteristic sequence of F3′H. This was because, in the amino acid sequence of the F3′H of *Oryza sativa* (japonica cultivar-group), "GGEK" was replaced by "GRMH".

All primers were synthesized by Invitrogen Biotechnology Co., Ltd..

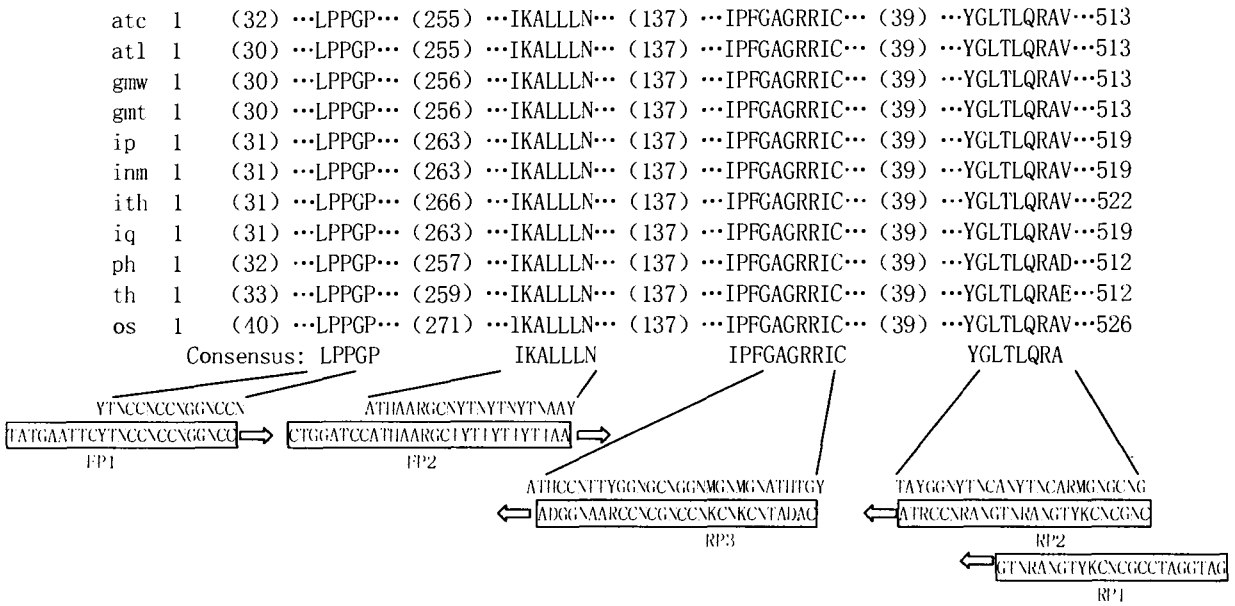

Fig. 2 Design of the degenerate primers used to amplify the segments of the *F3′Hs* from the 3 cultivars of *P. mume*

Top, the deduced amino acid sequences of 11 *F3′H* were aligned by DNassist 2.2. The four most highly conserved regions were shown, separated by the number of intervening amino acids (shown in parentheses). **Middle**, the consensus amino acids were shown below the aligned sequences. **Bottom**, the degenerate nucleotide sequences were determined according to part of the consensus amino acid sequences. FPs and RPs were designed and shown in the panes. The affixation of the restriction endonuclease sites of EcoR I or BamH I in the primers was used to adjust the Tms of the primers and administered to recognizing the PCR products in the pMD18-T vectors. Abbreviations: **atc**: *Arabidopsis thaliana* ecotype="Columbia"; **atl**: *Arabidopsis thaliana* ecotype="Landsberg erecta"; **gmw**: *Glycine max* 'Williams'; **gmt**: *Glycine max* 'To7B'; **ip**: *Ipomoea purpurea*; **inm**: *I. nil* Magen; **ith**: *I. tricolor* 'Heavenly Blue'; **iq**: *I. quamoclit*; **ph**: *Petunia hybrida*; **th**: *Torenia hybrida*; **os**: *Oryza sativa*.

Table 3 Primer design in degenerate PCR

Items	Primer name	Sequence(5′→3′)[a]	Degeneracy	Tm(℃)[c]	GC%(%)[c]
Forward primer	FP1	TATGAATTCYTNCCNCCNGGNCC [b]	512	56.0	43.5
	FP2	CTGGATCCATHAARGCIYTIYTIYTIAA [b]	48	52.3	25.0
Reverse primer	RP1	GATGGATCCGCNCKYTGNARNTG [b]	512	56.0	43.5
	RP2	CIGCICKYTGIARITGIARICCRTA	32	53.0	32.0
	RP3	CADATICKICKICCIGCICCRAAIGGDA	24	58.2	39.3

Note: [a] Y=C,T; N=A,G,C,T; H=A,C,T; R=A,G; K=G,T,D=A,G,T; I=inosine. [b] The underlined sequences designate restriction endonuclease site (GAATTC, *Eco*R I; GGATCC, *Bam*H I), and are used to adjust the Tms of the primers and to favor recognizing the PCR products in the pMD18-T vectors. [c] Tm and GC% are predicted and calculated by Introgen Biotechnology Co., Ltd.

2.3 Screening of the effective primers

In order to screen suitable primers to realize the efficient amplification of the segment of the *F3′H* from *P. mume*, 2 FPs and 3 RPs were combined to constitute total 6 sets of primer pairs in the degenerate PCR reactions. Pairs of FP and RP were selected or discarded according to the length comparison of the fragments obtained by PCR and those predicted by the deuced amino acid sequences which were spanned by the special pair of FP and RP.

It was found that, for all of the 3 cultivars of *P. mume*, only the primer set of FP2 and RP3 was suitable for amplifying the segment of the *F3′H* because only the length of the product amplified by FP2 and RP3 was longer than the theoretical length of the segment spanned by FP2 and RP3(Table 4).

2% agarose gel electrophoresis analysis indicated that, when the primer set of FP2 and RP3 was used in degenerate PCR, one about 470bp-length band was observed for all of the 3 cultivars(Fig. 3).

Table 4 Amplification results of degenerate PCR with different combinations of FP and RP

Combination of FP and RP	Approximate length of degenerate PCR product [a] (bp)	Theoretical length of the segment spanned by the pair of FP and RP [b] (bp)
FP1 and RP1	240	1 398
FP1 and RP2	450	1 398
FP1 and RP3	240,450 [c]	1 251
FP2 and RP1	440	603
FP2 and RP2	430	603
FP2 and RP3	470	462

Note: [a] The approximate lengths of the products amplified by degenerate PCR were determined by the comparison of the products with the bands produced by DNA marker. [b] The theoretical length of the segment spanned by the specific pair of FP and RP was calculated according to the amino acid number locating between the two sites where the FP and RP were designed. [c] Two obvious bands were observed on the electrophoretogram of the PCR products.

2.4 Efficiency of recombination and transformation

The recombination efficiency of the PCR products with the pMD18-T Vector was very high, being demonstrated by the fact that, in one LB-Amp plate, about 600 white colonies were found everywhere and only 3～7 blue colonies were observed.

The transformation of the above recombinant vector to *E. coli* DH5α competent cells was also very efficient. PCR inspection indicated that, in the 21 randomly selected white colonies, about 19. 4 colonies were positive ones averagely.

2.5 DNA sequence analysis

DNA sequencing revealed that the segments of the *F3′H*s of the 3 cultivars of *P. mume* are all 469 bp long.

A multiple alignment showed that they shared 99. 72% identity among each other(Fig. 4). Moreover, they also shared 65. 57% identity with the corresponding regions of the 11 cDNAs of the *F3′H*s which have been openly published and submitted in the GenBank databases(data not shown). However, the above alignments could not be used to completely verify the characteristics of the segments of the *F3′H*s, because so far, no isolation of a *F3′H* DNA clone had been reported.

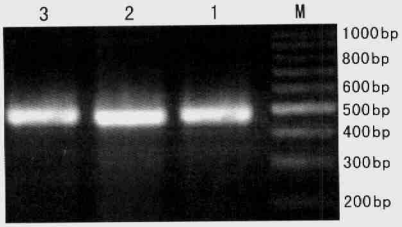

Fig. 3 2% agarose gel electrophoretogram of the three segments of the *F3′H*s amplified from the gDNAs of 3 cultivars of *P. mume*

Lane 1: *P. mume* 'Nanjing Hongxu'; **Lane 2**: *P. mume* 'Nanjing Hong'; **Lane 3**: *P. mume* 'Fenpi Gongfen'; **Lane M**: DNA marker. The concrete sizes of the bands produced by DNA marker were shown on the right.

3 Discussion

Searches against the GenBank revealed that no *F3′H* from ligneous plant had been cloned and 11 complete cDNAs of *F3′H*s had been openly published and submitted. In advance, the gDNAs were extracted from the tender leaves of *P. mume* 'Nanjing Hongxu', *P. mume* 'Nanjing Hong' and *P. mume* 'Fenpi Gongfen' with "Predeimpurity-SDS method" developed in this study. By aligning the 11 deduced amino acid sequences of the cDNAs, four most highly conserved amino acid regions were observed, from which 2 forward degenerate primers and 3 reverse degenerate primers were designed and consisted of 6 sets of primer pair. Only one primer pair could be used in degenerate PCR to amplify three 469bp segments from the gDNAs of

the 3 cultivars of *P. mume* respectively. Sequence analysis indicated that the 3 segments shared 99.72% identity among each other, and 65.57% identity with the corresponding regions of the 11 cDNAs. It is the first time for the segment of *F3′H* to be cloned from the gDNA of ligneous plant. Meanwhile, it was found that the "GGEK" was not the characteristic sequence of F3′H, which was not consistent with the previous viewpoint.

In degenerate PCR, it is impossible to foresee

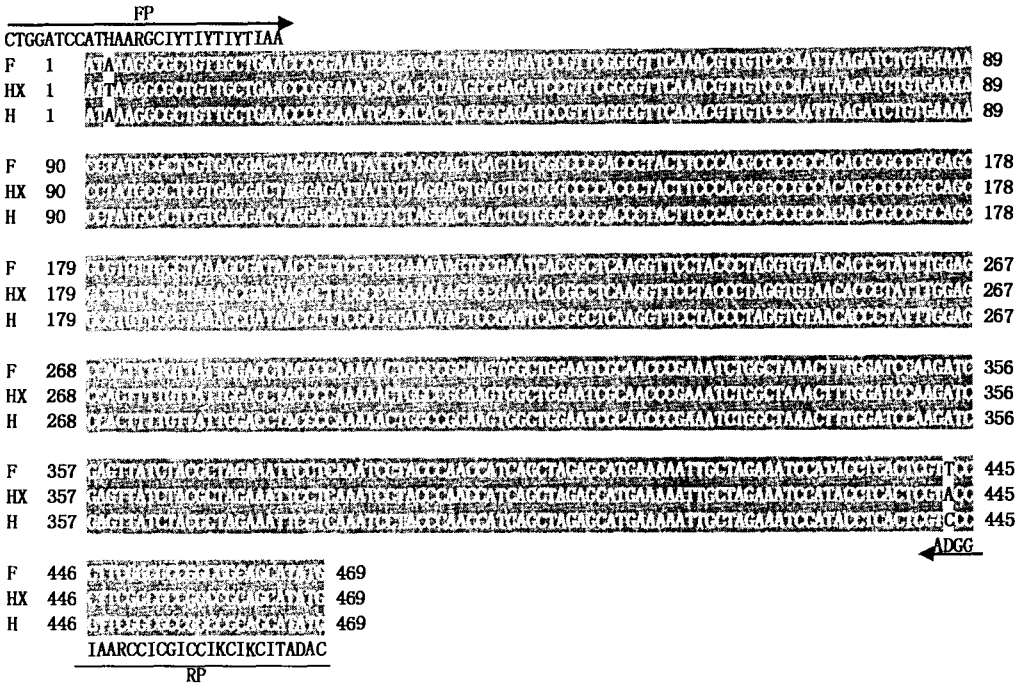

Fig. 4　Multiple alignment of the 3 segments of the *F3′H*s amplified from the gDNAs of 3 cultivars of *P. mume*

FP is shown by arrow above its corresponding sequence; **RP** is shown by arrow below its complementary sequence. Abbreviations: **F**: The segment from *P. mume* 'Fenpi Gongfen'; **H**: The segment from *P. mume* 'Nanjing Hong'; **HX**: The segment from *P. mume* 'Nanjing Hongxu'.

the efficiency of primers. It was found in this study that the targeted fragment might not be amplified by using theoretically reasonable degenerate primers. For example, the degenerate PCR in which the primer pair of FP1 and RP1 was used produced a strange short fragment which was consumedly shorter than the theoretically predicted length (data not shown). It may be because the conserved sequences by which the degenerate primers were designed are, by the square, be possessed by another genes. So it is always necessary to design more than one pair of degenerate primer to accomplish the demanded amplification.

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梅花类黄酮 3′-羟化酶基因片段基于基因组 DNA 的简并 PCR 法克隆

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摘 要：用该研究设计的"预先去杂—SDS 法"从梅花嫩叶提取到高质量的基因组 DNA。根据 11 条已公开发表的并提交到 GenBank 的类黄酮 3′-羟化酶基因 cDNA 的假定氨基酸序列的保守区设计 2 个正向简并引物和 3 个反向简并引物组成 6 对引物，仅有 1 对引物能以 PCR 法同时从梅花'南京红须'、'南京红'和'粉皮宫粉'的基因组 DNA 扩增到一个 469 bp 的核苷酸片段，这 3 个片段在总体上有 99.72%的一致性，与 11 条类黄酮 3′-羟化酶基因 cDNA 的相应区域有 65.57%的一致性。同时，"GGEK"并非类黄酮 3′-羟化酶的特征性模体。这是首次从木本植物的基因组 DNA 克隆到类黄酮 3′-羟化酶基因片段。该研究结果可为梅花类黄酮 3′-羟化酶基因全长的克隆奠定基础。

关键词：梅花；基因组 DNA 提取；类黄酮 3′-羟化酶基因片段；简并 PCR