Expressions and dimerization affinities of three highly identical *APETALA3* genes in *Brassica napus*

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Abstract

Three highly identical cDNA clones of *APETALA3* (*AP3*) gene, *BnAP3-2*, *BnAP3-3* and *BnAP3-4* were isolated from *Brassica napus* L. by RT-PCR. The sequence analysis showed that all the three *AP3* cDNAs contained a complete open reading frame. Their nucleotide sequences had 91 - 97 % similarity and their predicted amino acid sequences shared 93 - 98 % identity. Real-time quantitative RT-PCR result showed that all the three *BnAP3* genes were expressed at the transcriptional level in petals as well as stamens. Among the three *BnAP3* genes, *BnAP3-3* was expressed at the highest level and *BnAP3-2* was expressed at the lowest level in petals. The transcription level of *BnAP3-3* was 1.59 times than that of *BnAP3-2.* The transcription levels of *BnAP3-2*, *BnAP3-3* and *BnAP3-4* in stamen were 7.75, 5.11 and 3.88 times than those in petal, respectively. The yeast two-hybrid assays results showed that all the three BnAP3 proteins could form strong heterodimers with BnPI, and obviously different dimerization affinities among the three proteins to BnPI were observed. The ratio of the affinity of BnAP3-2, BnAP3-3 and BnAP3-4 to BnPI-1 was 1.27:1:1.62. Although the three *BnAP3* genes were highly identical, the differences of their expression and affinity of protein interaction might reflect some functional divergence.

Additional key words: gene duplication, rape, real-time quantitative PCR, yeast two-hybrid assays.

Introduction

Most angiosperm flowers consist of four organ types that are arranged in a series of concentric whorls. From outside to inside, the flower consists of sepals in whorl one, petals in whorl two, stamens in whorl three and carpels in whorl four. In *Arabidopsis thaliana* and *Antirrhinum majus*, the identity of floral organs is controlled by five classes of homeotic genes (ABCDE genes). The petal identity is determined by class A (*APETALA1*, *APETALA2*), class B (*APETALA3*, *APE3*), *PISTILLATA* (*PI*)) and class E (*SEPALLATA*). The stamen identity is determined by class B, class C (*AGAMOUS*) and class E (Jack 2001, Krizek and Fletcher 2005). Most floral homeotic genes encode members of the MADS family of transcription factors. Plant MADS proteins consist of four domains: the MADS (M) domain, a highly conserved DNA-binding domain; the I domain, an intervening region; the K domain, which is involved in protein-protein interactions; and the variable C domain, which may be involved in transcriptional activation and/or the formation of multimeric transcription factor complexes (Yang *et al*. 2003).

In *Arabidopsis, AP3* and *PI* are predominantly expressed in the second- and third-whorl floral organ primordia, and their expression is maintained until petals and stamens have fully developed. Mutations in either one of these B-class genes cause homeotic conversion of petals in the second whorl to sepals and of stamens in the third whorl to carpels (Bowman *et al.* 1989, 1991, Krizek and Meyerowitz 1996), which is consistent with their crucial role in petal and stamen development. The expression of either one of the B-function genes is initiated independently but that the maintenance of high levels of AP3 and PI depends upon the presence of the heterodimeric protein complex itself. The AP3/PI heterodimeric complex has been shown to be responsible for the continued expression of both AP3 and PI through a positive feedback loop (Jack *et al*. 1992, 1994, Goto and Meyerowitz 1994, Honma and Goto 2000). It was reported that heterodimerization between AP3 and PI was also

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Abbreviations: AP3 - APETALA3; PCR - polymerase chain reaction; *PI - PISTILLATA*; RT - reverse transcription.

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necessary for nuclear localization of these proteins (McGonigle *et al*. 1996).

Gene duplication occurs at a high rate in eukaryotic genomes in general (Lynch and Conery 2000) and in flowering plants in particular (Blanc and Wolfe 2004, Cui *et al*. 2006). It is hypothesized that gene duplication arising through polyploidy (whole-genome duplication) has played an important role in the evolution of diversity in flowering plants (Bodt *et al*. 2005, Duarte *et al*. 2005). Duplicated genes are generally considered to adopt one of three possible fates: nonfunctionalization (silencing of one copy), neofunctionalization (acquisition of a novel function for one copy), or subfunctionalization (partitioning of tissue-specific patterns of expression of the ancestral gene between the two copies; Lynch and Conery 2000). Gene duplication and diversification can provide the raw material for the evolution of new morphologies (Irish and Litt 2005). If a developmental pathway occurred in multiple, redundant genes might be more resistant to deleterious mutations than one with only a few such genes (Wagner 1998). Gene duplications are generally considered as a mechanism for increasing diversity and for functional innovation (Duarte *et al*. 2005).

Brassica napus is an amphidiploid species with the AACC genome originated from two diploid ancestral species: the A genome came from *B. rape*, and the B genome from *B. oleracea* (Nagahara 1935, Palmer *et al.* 1983, Parkin *et al*. 1995, Schmidt *et al*. 2001). Allopolyploidization leads to the generation of duplicated homoeologous genes. Therefore, many redundantly duplicated genes in *B. napus* might originate from the genomes of ancient ancestors, *B. rape* and *B. oleracea*, respectively. Pylatuik *et al*. (2003) isolated an *AP3* cDNA clone from *B. napus* and southern analysis suggested that up to three copies of *AP3* genes occurred in the *B. napus* genome.

In this study, we isolated the cDNAs of three highly identical *AP3* genes, *BnAP3-2*, *BnAP3-3* and *BnAP3-4*, from *B. napus* by RT-PCR. Three pair primers were designed according to the difference of single or a few nucleotides and real-time quantitative RT-PCR was used to detect the expression difference of the three *BnAP3* genes at the transcriptional level in petals and stamens. Finally, yeast two-hybrid assays were used to evaluate the dimerization affinities of the three AP3 proteins with a PI homolog in *B. napus*.

Materials and methods

Plants: An inbred line of *Brassica napus* L. (Zhao and Wang 2004) was cultivated in the test field of Sichuan University. The early floral buds (less than 200 μm in length) were collected under anatomical lens for isolation of the *AP*3 by RT-PCR. The petals and stamens of later floral buds (2 mm in length) were collected for real-time quantitative PCR. The recombinant plasmid pBnPI-1 was constructed by our group and contains the ORF cDNA of *BnPI-1* (EU159431).

Total RNA isolation, mRNA purification, and first-strand cDNA synthesis: Total RNA was isolated from above-mentioned tissues using *Trizol* reagent (*Invitrogen*, Carlsbad, CA, USA). The polyA mRNA was purified from total RNA with *PolyATract1System 1000* (*Promega*, Madison, WI, USA). First-strand cDNA synthesis was performed using 1 μg of purified mRNA obtained above, with oligo (dT) primer according to the protocol for RT-PCR first-strand synthesis (*Clontech*, Mountain View, CA, USA).

Isolation of full-length coding region of cDNAs: According to the registered sequence of a *B. napus AP3* cDNA (AF124814), a pair of primers, listed in Table 1 (primers I), were designed to amplify the entire coding region of *BnAP3* using *PrimerSTAR*^{TM} *HS* DNA polymerase (*TaKaRa Biotechnology*, Dalin, China). cDNA isolated from early floral buds was used as template. PCR reactions were performed using the following parameters: 94 °C for 5 min, 40 cycles at 94 °C for 45 s, 50 °C for 45 s and 72 °C for 45 s, and 72 °C for 5 min. PCR fragments were purified with the gel extraction mini kit, and the purified products were ligated with the *pMD18-T* vector (*TaKaRa Biotechnolgy*) to transform *E. coli* JM109 cells. 100 mm^3 of transformed cells were spread on LB plates containing 50 μg cm^{-3} ampicillin, 4 mm^3 isopropyl β-D-1-thiogalactopyranoside and 40 mm^3 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, and the plates were incubated overnight at 37 °C. Recombinant clones were sequenced in *Shanghai Sangon Biologic Engineering Technology and Service Co.* (Shanghai, China).

Identification of the specific primers for each AP3 gene: The recombinant plasmids with *BnAP3* cDNA were used as template at first to identify the specificity of each pair primers for each *AP3* gene. PCR reactions were performed using the following parameters: 95 °C for 5 min, 40 cycles at 95 °C for 45 s, 50/55/60 °C for 45 s and 72 °C for 45 s, and 72 °C for 5 min. PCR products were electrophoresed in a 1 % agarose gel.

Quantification of transcripts by real-time PCR: Real-time quantitative RT-PCR detection was performed with an *iCycler* machine and *iQTM SYBR* green supermix (*Bio-Rad,* Hercules, USA), and measured in a 96-well plate. For each well, the 25 mm^3 reaction contained 12.5 mm^3 of 2× *iQTM SYBR* green supermix, 0.5 μM each forward and reverse primer, 2.75 mm^3 of DNase-free H_2O, 3.75 mm^3 cDNA templates. PCR reactions were performed using the following parameters: 5 min at 95 °C, and 40 cycles of 45 s at 95 °C, 45 s at 60 °C and 45 s at 72 °C, and 72 °C for 5 min. PCR products were melted by gradually increasing the temperature from 60 to 95 °C in 0.5 °C steps. The mRNA levels for each of the detected

genes in different samples were computed with respect to the internal standard, β-actin, to normalize for variance in the quality of cDNAs. The one-way *ANOVA* method was used for statistics analysis. The gene-specific primers, P, P2, P3 and P4 (Table 1), were used for analysis of *BnAP3* gene expressions, respectively. The amplification efficiency of each primer and the specificity of the reaction were tested by melting curve analysis.

Yeast two-hybrid assays: Yeast two-hybrid experiments were conducted using the *Matchmaker GAL4* two-hybrid system 3 with bait and prey vectors (pGBKT7, pGADT7) containing the binding domain (BD) and activation domain (AD), respectively (*Clontech*, Mountain View, CA, USA). Yeast host strain Saccharomyces cerevisiae AH109 was used to take advantage of the four reporters ADE2, HIS3, *lacZ* and MEL1. The *lacZ* and MEL1 encode the *in vivo* β-galactosidase and secreted enzyme α-galactosidase, which hydrolyzes the colorless X-Gal and X-α-Gal into a blue compound, respectively. All yeast methods were conducted following manufacturer's instructions (*Clontech*).

The coding regions of each cDNA containing I, K and C region (IKC) of the *BnAP3-2* (61-223aa), *BnAP3-3* (61-231aa), *BnAP3-4* (61-223aa) and *BnPI-1* (61-208aa) were amplified by PCR. The PCR templates were the recombinant plasmids including coding regions of *BnAP3* and *BnPI* cDNA, respectively, and the profile used was 95 °C for 5 min, 35 cycles at 95 °C for 45 s, 55 °C for 45 s and 72 °C for 45 min, and 72 °C for 5 min, with the primers II and III (Table 1), respectively. The amplified sections of *BnAP3* were digested with *Eco*RI and *Pst*I, and cloned into pGBKT7, forming pBD-BnAP3 constructs. The amplified sections of BnPI were digested with *Eco*RI and *Bam*HI, and cloned into pGADT7, forming pAD-BnPI constructs. All constructs were sequenced to confirm that the binding domain or activation domain was fused N-terminally and in frame with the coding region of the appropriate locus. Transformation into yeast strain AH109 was performed with both constructs using a lithium acetate/PEG method following manufacturer's instructions (*Clontech*) or a kit (*Zymo Research Corp*, Orange, CA, USA). Strains were selected on synthetic dropout (SD) media supplemented with adenine (Ade) and 2 % glucose but lacking tryptophan (Trp) for the binding domain constructs and leucine (Leu) for the activation domain constructs. To check for auto-activation, four independent yeast transformants for each construct were tested for growth on media lacked either Trp for the BD constructs or lacking Leu for the AD constructs. The result shows that there is no auto-activation for all of them. Protein interactions were then tested by transformation of appropriate construct pairs into AH109 and growth on selective media. Selection for interactions were conducted on SD media lacking His, Leu, Trp (-HLT) or Ade, His, Leu, Trp (-AHLT).

Protein interactions were characterized using assays of both β-galactosidase (β-gal) and α-galactosidase (α-gal) activity. The *lacZ* marker was used to conduct β-gal assays. Experiments were conducted on agar plate. Fresh colonies were copied to a sterile filter presoaked by placing it in Z buffer/X-gal solution in a clean plate. They were immersed in liquid nitrogen for 10 s and then allowed to thaw at room temperature. After incubation on filters at 30 °C for 4 h blue colonies appeared. The positive colonies were transferred to fresh medium for the next assay of α-gal activity using MEL1 marker. Experiments were conducted on liquid SD media containing secreted α-gal and the substrate p-nitrophenyl α-D-galactopyranoside (PNPG) (*Sigma*, St. Louis, MO, USA). Methods are detailed in the Yeast Protocol Handbook (*Clontech*). For each experiment, three replicates per construct-pair were assessed. Cultures were grown overnight at 30 ºC shaking at 270 rpm. Each overnight culture was diluted to absorbance A_{600} of 0.8. 16 mm^3 of diluted culture was incubated with 48 mm^3 of assay buffer for 60 min at 30 ºC in darkness. Reaction was terminated with 136 mm^3 of stop solution. Absorbance of the solution was recorded at 410 nm. Activity of secreted α-gal was determined from the absorbance and appropriate dilution factors following calculations in the Yeast Protocol Handbook. The α-gal assays were repeated 3 times with three independent sets of yeast transformants for the entire set of protein interactions.

Results

Three duplicated AP3 genes: When RNA isolated from the early floral buds and primers I (Table 1) were used in RT-PCR, about 650 bp fragment was amplified (Fig. 1). The amplicon was purified with the gel extraction mini kit, ligated with the pMD18-T vector and the ligation was transformed into *E. coli* JM109 cells. Five recombinants were sequenced and three different sequences of *AP3* cDNAs were obtained after removing two repetitive cDNA clones. The sequence analysis showed that all the three *AP3* cDNAs, named *BnAP3-2* (AY313941), *BnAP3-3* (DQ372719) and *BnAP3-4* (DQ372720) respectively, contained a complete open reading frame, their nucleotide sequences had 91 - 97 % similarity and most of differential nucleotide sites were dispersed (Fig. 2). Their predicted amino acid sequences shared 93 - 98 % identity. Compared with the sequences of relative species in *Brassica* in GenBank, the nucleotide sequences of *BnAP3-2* and *BnAP3-4* share 99.56 and 96.59 % similarity with *B. oleracea AP3, Boi2AP3* (U67455), 96.74 and 100 % with *B. rapa AP3, BrAP3* (AY623003), respectively (Table 2). The nucleotide sequence of *BnAP3-3* shares 97.57 % identity with *B. oleracea AP3*, Boi1AP3 (U67453) (Table 2). The predicted amino acid sequences of the three *BnAP3* genes had much more identity with those of the relative species (Table 2).

Table 1. The primers used in this study. All primers are located in open reading frame (ORF). *Bold* - enzyme dissection sites, *italics* - protection nucleotides, *underline* - the differential nucleotides of the duplicated *BnAP3* genes.

Primer	cDNA fragment	Primer sequences	Amplicon [bp]
I	full-length coding region of *BnAP3*	up: 5'ATGGCGAGAGGGAAGATCC 3' down: 5' TTATTCAAGAAGGTGGAAGGT 3'	675 699
II	IKC region of *BnAP3*	up: 5'*CCG***GAATTC**AACACCACAACGAAGGAGATC3' down:5'*AAAA***CTGCAG**GTTATTCAAGAAGGTGGAAGGT3'	492 516
III	IKC region of *BnPI*	up: 5'*CCG***GAATTC**TCCATGGACCTTGGTGCTATG3' down: 5'*CGC***GGATCC**CCATCGATGACCAAAGACAT3'	447
P	Overall *BnAP3* genes	up: 5′ ATGGCGAGAGGGAAGATCC 3′ down: 5′ CGTCCAAACACTCACCTAGCCTC 3′	343
P2	*BnAP3-2*	up: 5' GCAAGAAACCAAGAGGAAACTGC 3' down: 5' GATGGTTCTGATGGAAACGAAG 3'	341
P3	*BnAP3-3*	up: 5' GCAAGAAACCAAGAGGAAGCTG 3' down: 5' AGCGTAAGCACGTGATCCTTC 3'	343
P4	*BnAP3-4*	up: 5' GCAAGAAACCAAGAGGAAATTA 3' down: 5' GATGGTTCTGATGGAAGCGAAG 3'	341

Table 2. The identity [%] of nucleotide and predicted amino acid sequences of *AP3* in *B. napus* and relative species (Bn - *Brassica napus,* Br - *B. rapa*, Bo - *B. oleracea,* nt - nucleotide sequences, aa - amino acid sequences.

Alignments		nt	aa
BnAP3-2	*Boi2AP3*	99.56	99.11
	BrAP3	96.74	98.22
	Boi1AP3	90.41	93.10
BnAP3-4	*BrAP3*	100.00	100.00
	Boi2AP3	96.59	98.21
	Boi1AP3	90.56	94.83
BnAP3-3	*Boi1AP3*	97.57	99.57
	BrAP3	90.84	94.40
	Boi2AP3	90.41	92.67

The specificity of the primers for each AP3 gene analyses: Primers P (Table 1) were designed and used to amplify the overall *BnAP3* genes. Three pair primers, P2, P3 and P4 (Table 1, Fig. 2), were designed according to the difference of single or a few nucleotides among the three *BnAP3* genes and used to amplify the specific single *BnAP3* gene in order to detect the differential expression of each redundant *BnAP3* gene. The recombinant plasmids with *BnAP3* cDNA were used as template at first to identify the specificity of the three pairs of primers for each *BnAP3* gene. The PCR results showed that the primers P could be used to amplify any of the three *BnAP3* genes at any annealing temperature tested. Only the specific segment (343 bp) of BnAP3-3 could be amplified at any of the three annealing temperature when P3 primers were used, indicating that P3 primers were very specific. But primers P2 and P4 were not specific when the annealing temperature is at 50/55 °C. Each of the three pair of primers could amplify specifically only one copy of the three *BnAP3* genes when the annealing temperature is up to 60 °C (Fig. 3). Furthermore, the cDNA of petals was used as template in real-time quantitative PCR and the melting curve was used to check the specificity of the gene-specific primers. When the universal primer P is used, melting curve is irregular, which shows that there are nonspecific products, although their very high degree of nucleotide sequence similarity. This means that all three *BnAP3* genes are amplified. When specific primers are used for each *BnAP3* gene, melting curve is regular, which means that there are only specific products. Each pair of primers could only amplify specifically one copy of the three *BnAP3* genes. All of these above indicated that the three pair of primers could be used in RT-PCR to analyze the differential expression of each redundant *BnAP3* gene when the annealing temperature is at 60 °C.

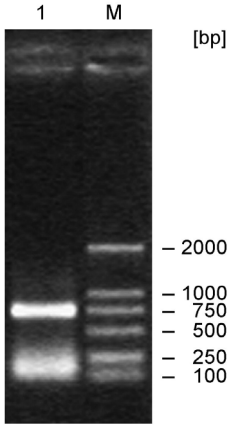

Fig. 1. The coding region of *AP3* cDNA in *B. napus* was amplified by RT-PCR. cDNA from early floral buds (less than 200 μm in length) and primers I (Table 1) were used. *Lane* 1 - amplicon of *AP3* cDNA, M - DNA marker DL 2000.

Expressions on transcription level of the duplicated BnAP3 genes: Gene-specific real-time quantitative RT-PCR was used to detect the differential expression of each redundant *BnAP3* genes in petals and stamens. It was observed that all the three *BnAP3* genes were expressed at

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BnAP3-2 ATGGCGAGAGGGAAGATCCAGATCAAGAGGATAGAGACCCAGACCAACCGACAAGTGACGTATTCAAAGAGAAGAAATGG 80
BnAP3-3 ****************************************A******************A**A*****C*************** 80
BnAP3-4 ****************************************A******A******************C*************** 80

BnAP3-2 TTTGTTCAAGAAAGCTCACGAGCTCACGGTCTTGTGTGACGCTAGGGTTTCGATTATCATGTTCTCTAGCTCCAACAAGC 160
BnAP3-3 ************************T*****T********T*********************************T******** 160
BnAP3-4 ********************************T*****************************************T********** 160

BnAP3-2 TTCATGAGTTTATCAGCCCTAACACCACAACGAAGGAGATCTTAGATCTGTACCAAACAGTTTCTGATGTTGATGTTTGG 240
BnAP3-3 **********C**T*****************A*********A****************C*****C*************** 240
BnAP3-4 ****************************************A************************************** 240
                                P2/P3/P4 up
BnAP3-2 AGCGCTCACTATGAGAGAATGCAAGAAACCAAGAGGAAACTGCTGGAGACAAATAGAAATCTTCGGACTCAGATTAAACA 320
BnAP3-3 *****A*********************************G***T*****************G**C**************G** 320
BnAP3-4 **T*********************************************T*AT************************************ 320

BnAP3-2 GAGGCTAGGCGAGTGTTTGGACGAGTTTGATATTCAGGAGCTGCGTAGTCTTGAGGAAGAAATGGAAAACACTTTCAAAC 400
BnAP3-3 *********T***************AC***************T************************************** 400
BnAP3-4 *********T********A*******C****************************************************** 400

BnAP3-2 TCGTTCGCGAGCGCAAGTTTAAATCGCTTGGGAATCAAATCGAGACCACCAAGAAAAAGAACAAGAGCCAGCAAGACATA 480
BnAP3-3 *******T*****************C***********G******A*********************A**T**A********** 480
BnAP3-4 ************************A********C*********************************T**A********** 480

BnAP3-2 CAAAAGAATCTCATACATGAGCTGGAACTAAGAGCAGAAGATCCTCATTATGGACTAGTAGACAATGGAGGAGACTACGA 560
BnAP3-3 ************************G*******************C******C****************C********* 560
BnAP3-4 ************************************************************************************ 560
                        P3 down                 P2/P4 down
BnAP3-2 TTCCGTTCTTGGATATCAA----------CTTCGTTTCCATCAGAACCATCACCACCATTATCCCA 616
BnAP3-3 ***G***************ATCGAAGGATCACGTGCTTACGCT*******A***************T*********C**** 640
BnAP3-4 ***A***************----------*****C*****************************C**** 616

BnAP3-2 ACCATGCCCTTCATGAAGCATCTGCCTCTGACATCATTACCTTCCACCTTCTTGAATAA 675
BnAP3-3 *****A**********C*C********************************************* 699
BnAP3-4 ***************C*G**************T******************************** 675
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Fig. 2. Alignments of nucleotide sequences of *BnAP3-2, BnAP3-3* and *BnAP3-4. Asterisk* (*) represents the same nucleotide and *dashes* (-) indicate gaps introduced to maximize alignment; *boxes* represent the location of P2, P3 and P4 primers.

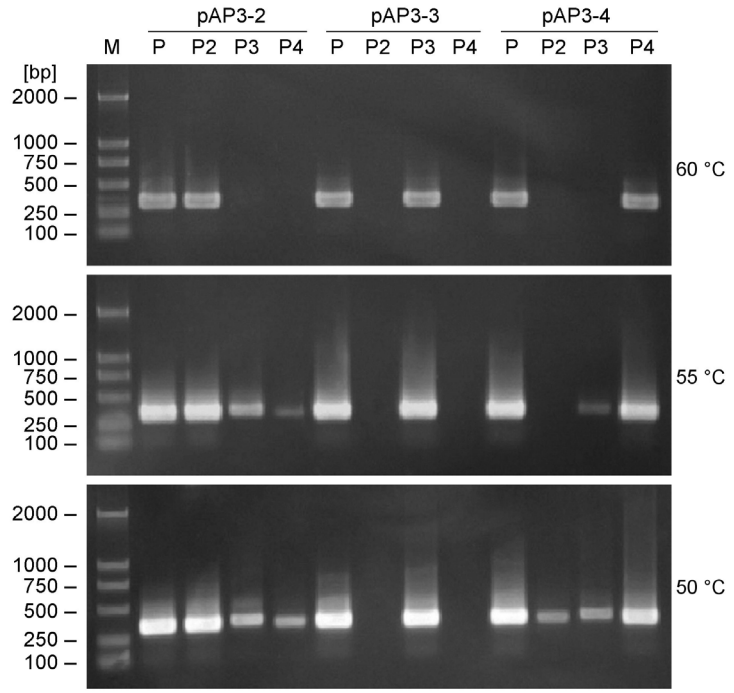

Fig. 3. The identification of the specific primers and the annealing temperature for each *AP3* gene. pAP3-2, pAP3-3 and pAP3-4 are recombinant plasmids containing BnAP3-2, BnAP3-3 and BnAP3-4, respectively. The three recombinant plasmids were used as template DNA, respectively. Primer P (Table 1) was used to amplify all the three BnAP3 segments. Three pair primers, P2, P3 and P4 (Table 1) were used to amplify the specific BnAP3 segment. From up to down, the annealing temperature is 60, 55 and 50 °C, respectively.

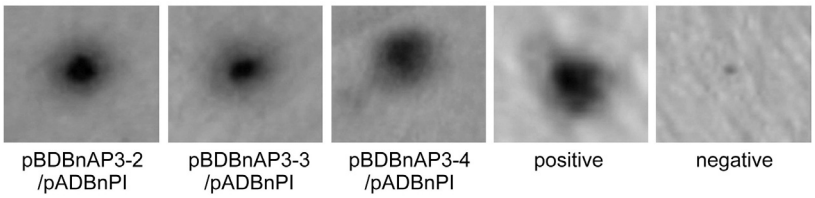

Fig. 4. Protein interactions between BnAP3 and BnPI homologs were detected with the *in vivo* β-galactosidase activity. Negative control: colonies with pGBKT-7 and pGBKT-7 vectors without any insertion sequence. Positive control: colonies with pGBKT-53 vector contain *p53* gene sequence and pGADT7-RecT vector contain *SV40* large T-antigen. p53 and SV40 large T-antigen could interact with each other in yeast cell. pBD-BnAP3/pAD-BnPI-1: colonies with pGBKT-BnAP3 vectors contain *BnAP3* sequences and pGADT7-BnPI-1 vectors contain *BnPI-1*.

the transcriptional level in petals as well as stamens. Among the three *BnAP3* genes, *BnAP3-3* was expressed at the highest level and *BnAP3-2* was expressed at the lowest level in petals. The transcription level of *BnAP3-3* was 1.59 times than that of *BnAP3-2*. The transcription level of *BnAP3-4* is 1.08 times than that of *BnAP3-2,* but statistical analysis showed that there is no significant difference between the expressions of *BnAP3-2* and *BnAP3-4*. However, the different expression patterns of the three genes were observed in stamen where each of the three single-AP3 genes was expressed significantly higher than that in petal. The transcription levels of *BnAP3-2*, *BnAP3-3* and *BnAP3-4* in stamen were 7.75, 5.11 and 3.88 times than those in petal respectively. Among the three *BnAP3* genes, *BnAP3-3* was expressed at the highest level and *BnAP3-4* was expressed at the lowest level in stamen. The transcription levels of *BnAP3-2* and *BnAP3-3* were 1.84 and 1.92 times than that of *BnAP3-4* respectively. In brief, the three *BnAP3* genes were all expressed and their expression patterns were different in petals and stamens.

Dimerization affinities to PI of the three BnAP3 proteins: When the full-length versions of BnAP3 and BnPI (MIKC) were used at first, no yeast colony grew on selective media. When the entire MADS domains (the first 60 amino acids) were eliminated, many yeast colonies were obtained on the selective media, which suggested that proper interaction was produced between BnAP3 and BnPI. At first, the β-gal activity was detected to confirm the interactions. The lacZ reporter gene encodes the *in vivo* β-gal, which hydrolyzes the colorless X-gal into a blue compound. When treated with X-gal, all yeast clones containing both pBD-BnAP3 and pAD-BnPI recombinant plasmids became into blue ones (Fig. 4), indicating that all of the three BnAP3 proteins could interact with BnPI. Further, assays of α-gal activity were performed to detect the interaction strength between the BnAP3 proteins with BnPI. The assays result of α-gal activity showed that all the three BnAP3 proteins could form strong heterodimers with BnPI and different dimerization affinities were found among the three BnAP3 proteins (Fig. 4). The affinity to BnPI-1 of BnAP3-4 is greater than that of BnAP3-2, while that of BnAP3-3 is the weakest. The statistical analysis showed that the affinity differences to BnPI-1among the three BnAP3 proteins were significant. The ratio of the affinity to BnPI-1 of BnAP3-2, BnAP3-3 and BnAP3-4 was 1.27:1:1.62.

Discussion

In the core eudicot models *Arabidopsis*, *Antirrhinum* and *Petunia*, AP3 and PI homologs have been found to form heterodimers, an interaction that is required for DNA binding as well as protein stability and nuclear import (Goto and Meyerowitz 1994, Jack *et al*. 1994, Zachgo *et al*. 1995, McGonigle *et al*. 1996, Riechmann *et al*. 1996a,b, Vandenbussche *et al.* 2004). Our yeast two-hybrid studies showed that all the three *B. napus* AP3 paralogs could form strong heterodimers with BnPI, much like their core eudicot homologs. However, the strength among the three duplicated BnAP3 paralogs was different. In addition, since there are also several duplicated *PI* genes in *B. napus*, we analyzed the dimerization affinities of each pair of the duplicated BnAP3 proteins to the duplicated BnPI proteins. To each BnPI protein, the duplicated BnAP3 proteins exhibit different affinities, meanwhile, for each of the BnAP3 proteins, the dimerization affinities among different BnPI proteins were also different (data not shown). Dimerization affinities differ among the duplicated BnAP3 paralogs and BnPI, indicating that particular AP3 proteins have greater affinity for specific PI gene products.

B. napus is an amphidiploid species formed from multiple independent fusion events between ancestors of the diploids *B. rapa* and *B. oleracea* (Nagahara 1935). Many genes might be duplicated in *B. napus,* so did *AP*3 genes. Gene duplication plays an important role in the evolution of diversity and novel function and is especially prevalent in the nuclear genomes of flowering plants. A duplicate copy can be released from constraint, enabling the evolution of new functions (neofunctionalization) or the loss of function (formation of a pseudogene). Alternatively, duplicated gene copies can potentially diverge in their roles, retaining different subfunctions of the original gene (Lewis 1951; Ohno 1970; Force 1999). Duplicate genes may be maintained through sub- and

neofunctionalization at the level of expression or coding sequence (Duarte *et al*. 2006). Maintenance of duplicated genes will be accompanied by divergence in expression or protein structure. Some of the subtle changes might represent evolutionarily "transient" states of truly redundant paralogs that will diverge functionally in the future (Duarte *et al.* 2006).

The *AP3* and *PI* lineage genes are thought to represent paralogous gene lineages that arose from a duplication event before the origin of the angiosperms. In addition, the *AP3* lineage underwent another major duplication event at the base of the core eudicots, giving rise to two *AP3* sublineages found in all groups of core eudicots: the *euAP3* and the *TM6* gene lineages (Kramer *et al*. 1998). The resulting two types of AP3-like proteins can easily be distinguished on the basis of their completely divergent C-terminal motifs, which have been named the paleoAP3 and euAP3 motifs. Interestingly, the euAP3 motif is exclusively found in AP3 proteins isolated from higher eudicots, whereas the paleoAP3 motif is encountered in AP3 proteins throughout the lower eudicots, magnoliid dicots, monocots and basal angiosperms. In addition, a number of higher eudicot species contain both the euAP3 and paleoAP3 type of genes (Kramer and Irish 2000). The divergence in sequence motifs of AP3 proteins has contributed to the evolution of divergent and distinct function for their functional protein (Lamb and Irish 2003). Some *AP3* duplicated genes found in either lower or higher eudicot exhibit distinct and complex expression patterns and may diverge in function (Vandenbussche *et al*. 2004, Kramer *et al*. 2007). In many plant species, more than one *AP3* and/or *PI* family member has been isolated, and most of these duplicated genes display differential functions. Gene duplications in both the *AP3* and *PI* lineages in *Petunia hybrida* have led to a functional diversification of their respective members, which is reflected by partner specificity and whorl-specific functions among these proteins (Vandenbussche *et al*. 2004). Detailed expression analyses of the three AP3 paralogs and one PI homolog in wild-type and floral homeotic mutant lines reveal complex patterns which suggest that canonical B class function has been elaborated in *Aquilegia* (Kramer *et al*. 2007). In *Oryza sativa*, two duplicated *PI/GLO*-like genes (*OsMADS2, OsMADS4*) were functional independent (Yadav *et al*. 2007). Our results showed that the three *AP3* genes, *BnAP3-2*, *BnAP3-3* and *BnAP3-4* have different expression patterns on transcription level in different organs in *B. napus*. Meanwhile, the dimerization affinities of BnPI to the three proteins, BnAP3-2, BnAP3-3 and BnAP3-4, are also different. Although all of the three *AP3* genes are highly identical and belong to the euAP3 lineage of core eudicots, the divergences of expression and affinity of protein interaction might reflect some functional divergence of the three *AP3* genes in *B. napus*.

It is very important to detect the differential expression of duplicated genes on transcriptional level. However, it is difficult to distinguish highly homologous duplication genes. It is apparently that Northern blot and *in situ* hybridization based on homologous sequence can not be used to detect the expression of highly identical duplicated genes. Analyzing similarity and difference of *cis*-regulatory elements of duplicated genes on a genome scale might reveal some expression information, but it needs a vast amount of bio-information data about duplicated genes. Our data suggest that real-time quantitative RT-PCR is an effective method to detect the expression difference of highly identical duplicated genes with the gene-specific primers designed according to the difference of single or a few nucleotides.

Reference

Blanc, G., Wolfe, K.H.: Widespread paleopolyploidy in model plant species inferred from age distributions of duplicate genes. - Plant Cell **16**: 1667-1678, 2004.

Bodt, S., Maere, S., Peer, Y.V.: Genome duplication and the origin of angiosperms. - Trends Ecol. Evol. **20**: 591-597, 2005.

Bowman, J.L., Smyth, D.R., Meyerowitz, E.M.: Genes directing flower development in *Arabidopsis*. - Plant Cell **1**: 37-52, 1989.

Bowman, J.L., Smyth, D.R., Meyerowitz, E.M.: Genetic interactions among floral homeotic genes of *Arabidopsis*. - Development **112**: 1-20, 1991.

Cui, L., Wall, P.K., Leebens-Mack, J.H., Lindsay, B.G., Soltis, D.E., Doyle, J.J., Soltis, P.S., Carlson, J.E., Arumuganathan, K., Barakat, A., Albert, V.A., Ma, H., De Pamphilis, C.W.: Widespread genome duplications throughout the history of flowering plants. - Genome Res. **16**: 738-749, 2006.

Duarte, J.M., Cui, L., Wall, P.K., Zhang, Q., Zhang, X., Leebens-Mack, J., Ma, H., Altman, N., De Pamphilis, C.W.: Expression pattern shifts following duplication indicative of subfunctionalization and neofunctionalization in regulatory genes of *Arabidopsis*. - Mol. Biol. Evol. **23**: 469-478, 2006.

Force, A., Lynch, M., Pickett, F.B., Amores, A., Yan, Y.L., Postlethwait, J.: Preservation of duplicate genes by complementary, degenerative mutations. - Genetics **151**: 1531-1545, 1999.

Goto, K., Meyerowitz, E.M.: Function and regulation of the *Arabidopsis* floral homeotic gene *PISTILLATA*. - Genes Dev. **8**: 1548-1560, 1994.

Honma, T., Goto, K.: The *Arabidopsis* floral homeotic gene *PISTILLATA* is regulated by discrete cis-elements responsive to induction and maintenance signals. - Development **127**: 2021-2030, 2000.

Irish, V.F., Litt, A.: Flower development and evolution: gene duplication, diversification and redeployment. - Curr. Opin. Genet. Dev. **15**: 454-460, 2005.

Jack, T.: Relearning our ABCs: new twists on an old model. - Trends Plant Sci. **6**: 310-316, 2001.

Jack, T., Brockman, L.L., Meyerowitz, E.M.: The homeotic gene *APETALA3* of *Arabidopsis thaliana* encodes a MADS box and is expressed in petals and stamens. - Cell **68**: 683-697, 1992.

Jack, T., Fox, G.L., Meyerowitz, E.M.: *Arabidopsis* homeotic gene *APETALA3* ectopic expression: transcriptional and

posttranscriptional regulation determine floral organ identity. - Cell **76**: 703-716, 1994.

Kramer, E.M., Dorit, R.L., Irish, V.F.: Molecular evolution of genes controlling petal and stamen development: duplication and divergence within the *APETALA3* and *PISTILLATA* MADS-box gene lineages. - Genetics **149**: 765-783, 1998.

Kramer, E.M., Holappa, L., Gould, B., Jaramillo, M.A., Setnikov, D., Santiago, P.M.: Elaboration of B gene function to include the identity of novel floral organs in the lower eudicot *Aquilegia*. - Plant Cell **19**: 750-766, 2007.

Kramer, E.M., Irish, V.F.: Evolution of the petal and stamen developmental programs: evidence from comparative studies of the lower eudicots and basal angiosperms. - Int. J. Plant Sci. **16**: 29-30, 2000.

Krizek, B.A., Fletcher, J.C.: Molecular mechanisms of flower development: an armchair guide. - Nature **42**: 688-698, 2005.

Krizek, B.A., Meyerowitz, E.M.: The *Arabidopsis* homeotic genes *APETALA3* and *PISTILLATA* are sufficient to provide the B class organ identity function. - Development **122**: 11-22, 1996.

Lamb, R.S., Irish, V.F.L.: Functional divergence within the *APETALA3*/*PISTILLATA* floral homeotic gene lineages. - Proc. nat. Acad. Sci. USA **100**: 6558-6563, 2003.

Lewis, E.B.: Pseudoallelism and gene evolution. - Cold Spring Harb. Symp Quant. Biol. **16**: 159-174, 1951.

Lynch, M., Conery, J.S.: The evolutionary fate and consequences of duplicate genes. - Science **290**: 1151-1155, 2000.

McGonigle, B., Bouhideland, K., Irish, V.F.: Nuclear localization of the *Arabidopsis APETALA3* and *PISTILLATA* homeotic gene products depends on their simultaneous expression. - Genes Dev. **10**: 1812-1821, 1996.

Nagahara, U.: Genome analysis in *Brassica* with special reference to the experimental formation of *B. napus* and peculiar mode of fertilisation. - J. jap. Bot. **7**: 389-452, 1935.

Ohno, S.: Evolution by Gene Duplication. - Springer-Verlag, Berlin - New York 1970.

Palmer, J.D., Shields, C.R., Cohen, D.B., Orton, T.J.: Chloroplast DNA evolution and the origin of amphidiploid *Brassica* species. - Theor. appl. Genet. **65**: 181-189, 1983.

Parkin, I.A.P., Sharpe, A.G., Keith, D.J., Lydiate, D.J.: Identification of the A and C genomes of the amphidiploid *Brassica napus* (oilseed rape). - Genome **38**: 1122-1131, 1995.

Pylatuik, J.D., Lindsay, D.L., Davis, A.R., Bonham-Smith, P.C.: Isolation and characterization of a *Brassica napus* cDNA corresponding to a B-class floral development gene. - J. exp. Bot. **54**: 2385-2387, 2003.

Riechmann, J.L., Krizek, B.A., Meyerowitz, E.M.: Dimerization specificity of *Arabidopsis* MADS domain homeotic proteins *APETALA1*, *APETALA3*, *PISTILLATA*, and *AGAMOUS*. - Proc. nat. Acad. Sci. USA **93**: 4793-4798, 1996b.

Riechmann, J.L., Wang, M., Meyerowitz, E.M.: DNA-binding properties of *Arabidopsis* MADS domain homeotic proteins *APETALA1*, *APETALA3, PISTILLATA* and *AGAMOUS*. - Nucl. Acids Res. **24**: 3134-3141, 1996a.

Schmidt, R., Acarkan, A., Boivin, K.: Comparative structural genomics in the *Brassicaceae* family. - Plant Physiol. Biochem. **39**: 253-262, 2001.

Vandenbussche, M., Zethof, J., Royaert, S., Weterings, K., Gerats, T.: The duplicated B-class heterodimer model: whorl specific effects and complex genetic interactions in *Petunia hybrida* flower development. - Plant Cell **16**: 741-754, 2004.

Wagner, A.: The fate of duplicated genes: loss or new function? - Bioessays **20**: 785-788, 1998.

Yadav, S.R., Prasad, K., Vijayraghavan, U.: Divergent regulatory *OsMADS2* functions control size, shape and differentiation of the highly derived rice floret second whorl organ. - Genetics **176**: 283-294, 2007.

Yang, Y., Fanning, L., Jack, T.: The K domain mediates heterodimerization of the *Arabidopsis* floral organ identity proteins, *APETALA3* and *PISTILLATA*. - Plant J. **33**: 47-59, 2003.

Zachgo, S., Andrade, S.E., Motte, P., Trobner, W., Saedler, H., Schwarz-Sommer, Z.: Functional analysis of the *Antirrhinum* floral homeotic deficiens gene *in vivo* and *in vitro* by using a temperature-sensitive mutant. - Development **121**: 2861-2875, 1995.

Zhao, Y., Wang, M.L.: Inheritance and agronomic performance of an apetalous flower mutant in *Brassica napus* L. - Euphytica **137**: 381-386, 2004.