March 2016. Horticultural Plant Journal, 2 (2): 91–96.

Horticultural Plant Journal

Available online at www.sciencedirect.com
The journal's homepage: http://www.journals.elsevier.com/horticultural-plant-journal

PAP3 Regulates Stamen but Not Petal Development in *Capsicum annuum* L.

MA Ning, LIU Chen, YANG Wencai, and SHEN Huolin *

Beijing Key Laboratory of Facilities Vegetables Growth Regulation, College of Horticulture, China Agricultural University, Beijing 100193, China

Received 1 December 2015; Received in revised form 15 January 2016; Accepted 1 March 2016

Available online 14 June 2016

Abstract

Pepper flowers are hermaphroditic; the plant's male sterility trait is characterized by its inability to produce pollen grains. In the ABC model of flower development, B-function genes play roles in petal and stamen development in the angiosperm. In this study, a B-class gene designated as *PAP3* (GenBank accession no. HM104635) was isolated in pepper. The gene encoded 226 amino acids and shared high similarity with the MADS-box protein family, with a conservative MADS domain and semiconservative K domain. Furthermore, the expression of *PAP3* was abundant only in petals and anthers but not in leaves. A functional study employing virus-induced gene silencing (VIGS) showed that knockdown of *PAP3* led to the shriveling of pollen grains and male sterility; however, it did not affect petal development. These results suggest an essential role for *PAP3* in the development of the pepper stamen and in contributing to the variation of floral traits.

Keywords: *Capsicum annuum*; *PAP3*; MADS-box protein; VIGS; flower development; male sterility

1. Introduction

The identification of MADS-box gene has inspired studies of the genetic and molecular mechanisms that shape the identities of the various floral organs (Shinozuka et al., 1999). The functions of MADS-box transcription factor genes were summarized in the ABC model, which was built on genetic studies of floral development in *Arabidopsis* and *Antirrhinum* (Bowman et al., 1991; Coen and Meyerowitz, 1991; Ma and de Pamphilis, 2000). Floral organ genes have 3 classes of functions: A-function genes alone yield sepals, A-function genes in combination with B-function genes yield petals, B-function genes with C-function genes yield stamens, and C-function genes alone yield carpels. The history of the MADS-box gene family is characterized by the loss and duplication of genes (Hileman et al., 2006; Leseberg et al., 2006). The proteins of floral MADS-box genes regulate the genes' expression by forming multiple complexes.

Two B-function genes of the MADS-box gene family, *APETALA3* (*AP3*)/*DEFICIENS* (*DEF*) and *GLOBOSA* (*GLO*)/*PISTILLATA* (*PI*), are conserved transcriptional regulators that are needed to determine the identities of petals and stamens in angiosperms species (Becker and Theissen, 2003; Whipple et al., 2004). In *Arabidopsis*, mutations of *AP3* and *PI* can convert petals and stamens to sepals and carpels respectively. Overexpression of the *Chloranthus spicatus* gene *CsAP3* in *Arabidopsis* rescues stamen development only partially and does not affect petal development (Su et al., 2008). Four distinct domains are present in *AP3/DEF*: the MADS domain (M), the intervening region (I), the keratin-like domain (K), and the C-terminus (C) (Münster et al., 1997). Based on the characteristic structure of its C-terminal regions, the *AP3/DEF* lineage can be divided into 2 sublineages, eu*AP3* and *TOMATOMADS-BOX GENE6* (*TM6*) (Kang et al., 1998; Hernandez-Hernandez et al., 2007). *TM6*-type genes exist in a broad range of the angiosperms species, underscoring their conserved nature and ubiquity (Pnueli et al., 2008).

Pepper (*Capsicum annuum* L.) is one of the most widely consumed vegetables in the world owing to its unique sensory attributes of color, pungency, and flavor (Andrews, 1995). Most seeds used in pepper production are hybrids. The male sterility system in hybrid seed production can eliminate the cost of emasculation and ensure the purity of hybrid seeds by preventing self-pollination. Two types of male sterility, cytoplasmic male sterility (CMS) and genic male sterility (GMS), have been used in certain cultivars of pepper for seed production, and genes relevant to pollen formation and germination in GMS have been investigated in recent years (Chen et al., 2011). The CMS is more useful

* Corresponding author. Tel.: +86 10 82109512
E-mail address: SHL1606@cau.edu.cn

Peer review under responsibility of Chinese Society for Horticultural Science (CSHS) and Institute of Vegetables and Flowers (IVF), Chinese Academy of Agricultural Sciences (CAAS)

http://dx.doi.org/10.1016/j.hpj.2016.02.009

than the GMS system in the production of hybrid pepper seeds because it is maternally inherited and has certain mitochondrially associated traits (Lee et al., 2010). Furthermore, the CMS system has an obvious advantage in that it ensures 100% sterility in the female parent. *Rf* genes can be introduced from hot pepper to sweet pepper lines by repeated backcrossing. The detailed molecular mechanism of CMS in pepper remains unclear, although the CMS mitochondrial factors and CMS-associated genes have been identified (Kaufmann et al., 2005; Kim et al., 2007).

We recently identified a fragment TA288 from an EST library constructed for the identification of genes that are differentially expressed in near-isogenic CMS lines and their corresponding restorer lines in pepper (Guo et al., 2009a). By blasting this fragment against the EST database of the National Center for Biotechnology Information (NCBI), this fragment was found to have great similarity (95%) to *AP3* genes. In the current study, we isolated the gene designated as *PAP3* and investigated its function during flower development in pepper. The results offer some fundamental guidance for investigating the mechanism of male sterility in CMS lines in pepper.

2. Materials and methods

2.1. Plant material

Two pepper lines, 121A and 121C, were used in this study. The CMS line 121A was developed by backcrossing '8907A', an elite breeding line with CMS sterility, to an inbred hot pepper line 121. To obtain its corresponding restorer line, 121C, the line 8907A was backcrossed to a Chinese landrace 'Dajintiao' containing a single-gene dominant restoring gene (Guo et al., 2009b). Plants of lines 121A and 121C were grown in a greenhouse. Anthers, leaves, petals, sepals, and ovaries were collected, immediately frozen in liquid nitrogen, and stored at −80 °C until DNA or RNA extraction. Anthers were collected after the corolla became white. The microspore development was divided in to 4 consecutive stages: tetrad, early- or mid-uninucleate, late-uninucleate, and binucleate, as described in Willcox et al. (1991).

2.2. DNA/RNA isolation

Genomic DNA was extracted from young leaves using the CTAB method. Total RNA was extracted from 5 organs using the SV Total RNA Isolation System Kit (Promega, USA) according to the manufacturer's protocol. The total RNA was treated with RNase-free DNase. The RNA concentration was determined on 1.2% denaturing agarose gels, stained with ethidium bromide, and photographed using a GIAS-4400 Gel Documentation System (Beony Science and Technology Co., Beijing, China). The RNA was then used to amplify *PAP3* and semiquantitative RT-PCR.

2.3. Amplification and sequence analysis of the PAP3 gene in pepper

The full-length cDNA of *PAP3* was obtained using the rapid amplification of cDNA ends (RACE) technology. The first-strand cDNA was synthesized using the TaKaRa cDNA synthesis kit (TaKaRa, Japan) following the manufacturer's instructions. 3′ RACE and 5′ RACE were performed using the SMARTer RACE cDNA Amplification Kit (Clontech, USA) following the manufacturer's instruction.

The PCR products were separated on 1.5% agarose gel, recovered using Gel Extraction Kit (TIANGEN, China), and sequenced at Sunbiotech (Beijing, China). ORF finder from NCBI was used to deduce the amino acid sequence. The putative domain was predicted by SMART (http://smart.embl-heidelberg.de/). Amino acid sequences of *AP3/DEF* from 6 additional species were downloaded from NCBI and subjected to multiple-alignment using DNAMAN. The MADS-box genes from 5 subfamilies (Theissen et al., 2000) in *Arabidopsis thaliana* were downloaded from NCBI for phylogenic analysis of *PAP3* gene using the MEGA 4 program.

2.4. RT-PCR and Northern blot analysis

For semiquantitative RT-PCR, the first-stranded cDNA was synthesized from the 5 organs using M-MLV Reverse Transcriptase (Promega, USA) following the manufacturer's protocol. The products were standardized for semiquantitative RT-PCR by using an *Actin* gene (GenBank accession No. GQ339766.1) as the internal control.

For Northern blot analysis, total RNA was extracted from the flower buds at 4 stages using TRIzol reagents (Invitrogen, USA). The gel was stained with ethidium bromide, digitally photographed, and then fractionated and transferred to a Hybond N+ nylon membrane by capillary blotting. Northern blot hybridization was performed at 64 °C in an efficient hybridization solution. A fragment of *PAP3* amplified from pepper cDNA was used as the probe. Detection of the DIG label was done using the DIG Luminescent Detection Kit (Mylab, China) following the manufacturer's protocol.

2.5. Virus-induced gene silencing (VIGS) for PAP3

VIGS via the *Tobacco rattle virus* (TRV) system was used to knock down the expression of *PAP3* in pepper. The pTRV1 and pTRV2 VIGS vectors (Liu et al., 2002) were kindly provided by Dr. Yule Liu at Tsinghua University. To obtain the plasmid construct pTRV2-*pPAP3*, a 361 bp fragment of *PAP3* was amplified by PCR from pepper 121C cDNA with *Eco*R I and *Bam*H I restriction sites. The products were ligated into the pTRV2 vector and verified through sequencing. The plasmid construct TRV-*pPAP3* was transformed into *Agrobacterium tumefaciens* GV3101. The induced *Agrobacterium* mixtures of pTRV1 and pTRV2, pTRV2-*pPDS* or pTRV2-*pPAP3* (1:1 ratio) were infiltrated into the lower leaves of 4-leaf-stage plants of the line 121C using a syringe (Chung et al., 2004; Liu et al., 2004).

Stamen size, presence of pollen, and style length were monitored after leaves of the plants transformed with pTRV2-*pPDS* turned white. The pollen from wild-type, pTRV2 control, and the VIGS-silenced plants were dyed with I-KI solution and observed under a biological microscope. Two anthers were observed on one glass slide, showing the number of normally developed pollens by 40× objective. Total RNA was isolated from the anthers of the silenced and nonsilenced pepper plants for RT-PCR using the methods described above.

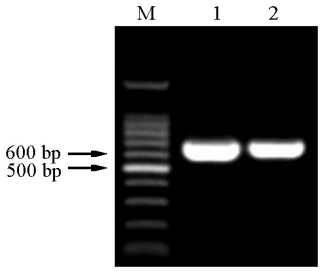

Fig. 1 Agarose gel image of PCR products for the *PAP3* gene amplified from genomic DNA and cDNA in CMS line 121C in pepper
Lane 1: cDNA; Lane 2: Genomic DNA; M: 100 bp marker.

3. Results

3.1. Cloning and sequence analysis of PAP3 gene

Using primers specific to the EST fragment of TA288, fragments of 752 bp and 625 bp were obtained through 3′ RACE and 5′ RACE respectively. The 3 fragments were assembled into a 929 bp sequence with a 678 bp open reading frame; they were designated as *PAP3* (GenBank accession no. HM104635). Amplification of genomic DNA and cDNA using the primer pair resulted in PCR products of the same size (Fig. 1). The complete open sequence encoded a putative 75.9 kD protein of 226 amino acids with an isoelectric point of 4.97. The deduced protein contained 2 conserved domains, a typical MADS-box (amino acids 2–61), an N terminus, and a K-box (amino acids 86–176) in the middle region, suggesting that the *PAP3* gene belonged to the MADS-box gene family.

The deduced amino acid sequence of *PAP3* was homologous to a large number of *AP3/DEF* related deduced proteins or unknown proteins with greater than 72% identity and more than 95% sequence coverage. In particular, it shared an identity of 91% with the tomato (*Solanum lycopersicum*) TAP3 protein (GenBank accession no. NM_001247148). Alignment of the PAP3 with 6 *AP3/DEF* genes showed that all *AP3/DEF* had a typical MADS-box N-terminus and a K-box domain. The 5′ end was more conserved than the 3′ end (Fig. 2). The phylogenetic tree showed that PAP3 was closely related to AP3 (NP_191002) in *Arabidopsis*, and they were clustered together in the AP3/PI group (Fig. 3).

3.2. Expression of PAP3

Semiquantitative RT-PCR analysis showed that *PAP3* expression was higher in 121C than 121A (Fig. 4, A). Moreover, the expression of *PAP3* was highest in anthers followed by petals

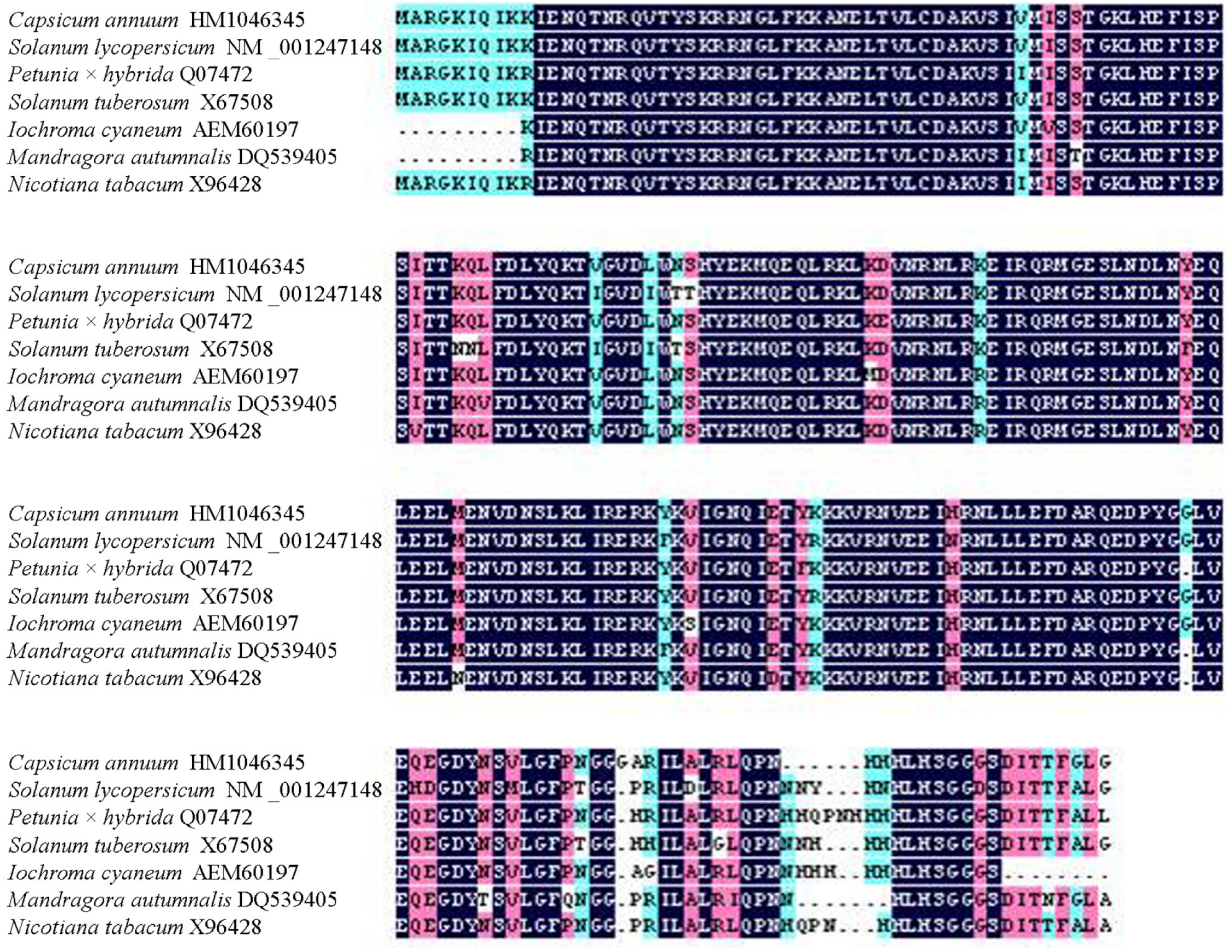

Fig. 2 Alignment of the deduced amino-acid sequences of *PAP3* with other *AP3/DEF* genes
Conserved amino acids are shaded in black and similar amino acids are shaded in gray. Gaps are indicated by solid circles.

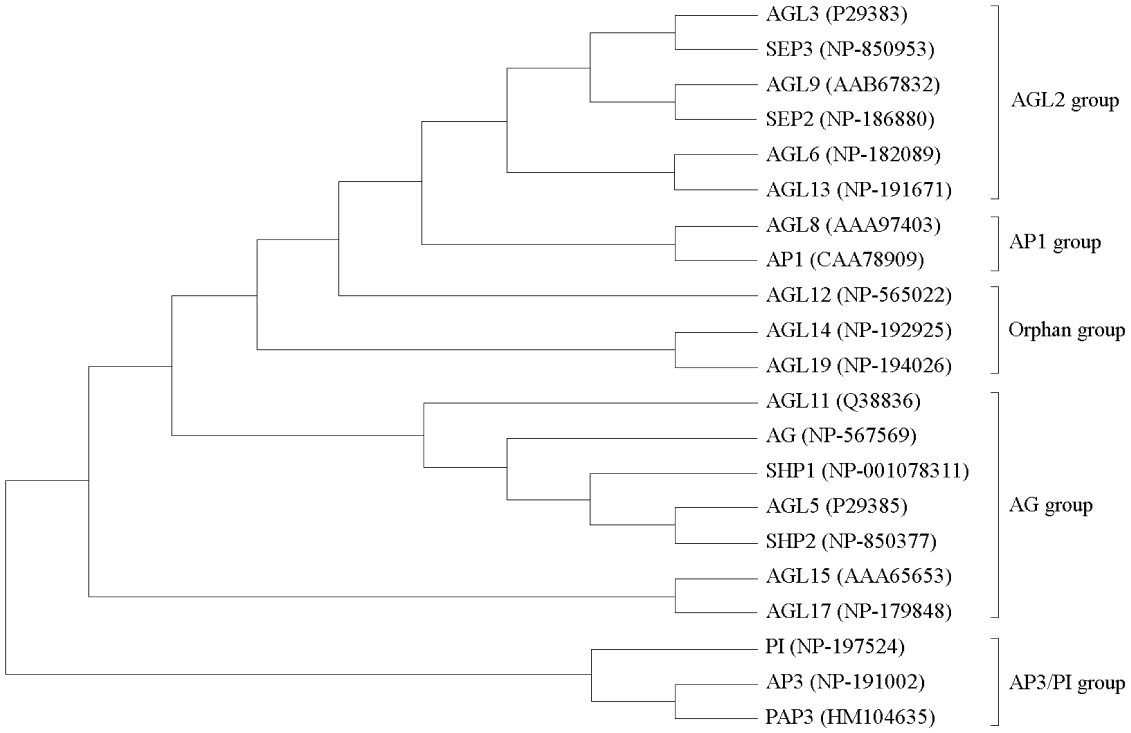

Fig. 3 Phylogenetic tree analysis of PAP3 and related proteins

but low in both sepals and ovaries. No expression was detected in leaves (Fig. 4, B). These results indicate that expression of the *PAP3* gene was similar to that of the B-function genes of the MADS-box gene family (María et al., 2007). Northern bolt analysis showed that *PAP3* was expressed at all 4 stages of anther development (Fig. 4, C).

3.3. Inhibition of PAP3 gene expression affects stamen but not petal development in pepper

All plants showed normal vegetative growth and flowered normally within 50 days after infection with pTRV2-*pPAP3* through Agro-infiltration. However, 28% (14 of 50) of pTRV2-*pPAP3* plants showed typical male sterility characteristics, such as shriveled anthers, long styles, and almost no pollen after 50 days of Agro-infiltration (Fig. 5), whereas petals developed normally in the whole growth process. No obvious phenotypic changes were observed in control plants.

The efficiency of gene silencing was examined by semiquantitative RT-PCR using the white corolla anthers of wild type, pTRV2 control, and silenced pepper plants. The results showed that expression of the *PAP3* gene in pTRV2-*pPAP3* plants was obviously reduced as compared with the pTRV2 control plants (Fig. 6).

Meanwhile, pollens of both the pTRV2-*pPAP3*-silenced plants and control plants showed significant differences under the microscope. On average, we found 18 and 16 normal pollens in wild-type and pTRV2 control plants respectively based on 10 fields of view under the microscope. In contrast, normally developed pollens were not detected in pTRV2-*pPAP3* silenced plants (Fig. 7). These results again suggest that *PAP3* plays an essential role in the development of pollen in the pepper plant.

4. Discussion

Many MADS-box genes remained largely uncharacterized in pepper, whereas they attract much attention in other plant

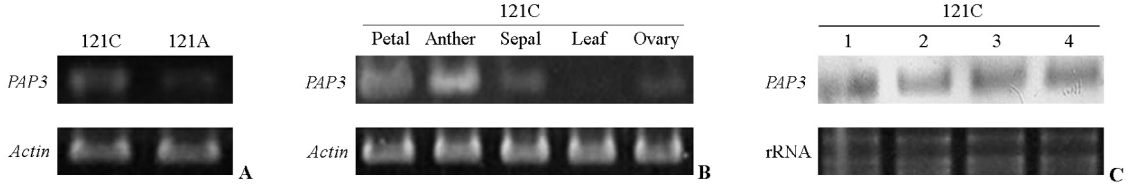

Fig. 4 Expression of *PAP3* in pepper

(A) RT-PCR analysis of the *PAP3* gene in restorer line 121C and male sterility line 121A; (B) RT-PCR analysis of the *PAP3* gene in different tissues of the restorer line 121C; (C) expression of the *PAP3* gene in the flower bud; four stages of microspore formation in line 121C detected by Northern bolt; 1. Tetrad; 2. Early- or mid-uninucleate; 3. Late-uninucleate; 4. Binucleate stages.

Fig. 5 Morphology of the floral organ
(A) The pTRV2 control plant with a normal flower; (B) a flower with shriveled anthers in a pTRV2-*pPAP3* silenced plant; (C) flower buds in control and silenced plants. The style is longer in the silenced plants than in the control plant.

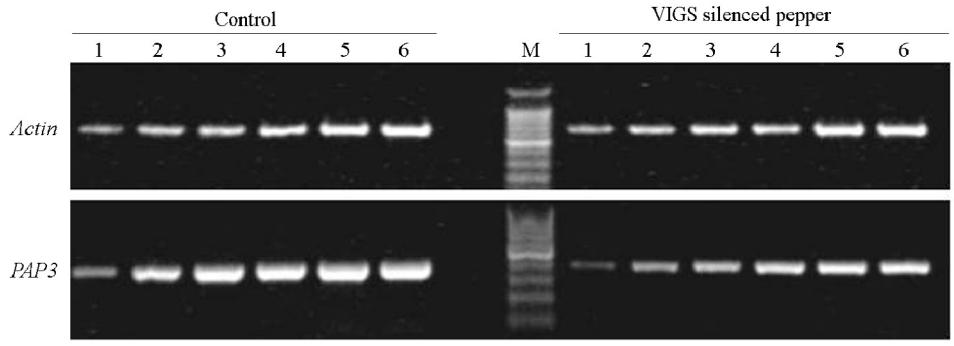

Fig. 6 Expression of *PAP3* in control and VIGS silenced pepper plants
Lanes 1 to 6 correspond to the product from PCR cycle numbers 15, 18, 21, 24, 27, and 30. M represents the 100 bp marker.

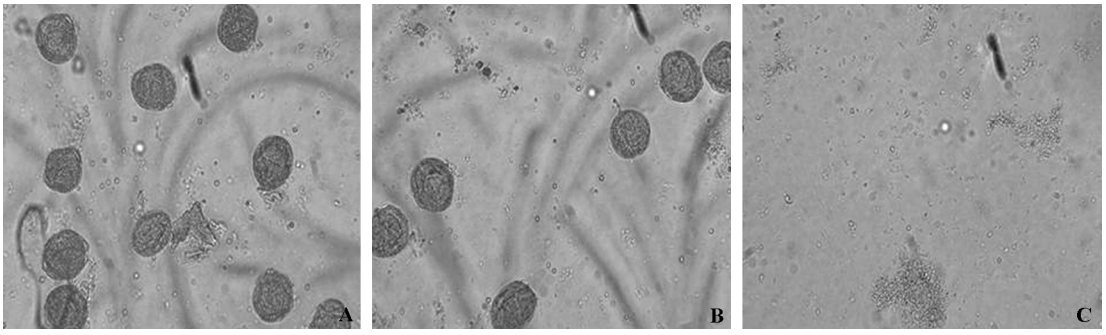

Fig. 7 View of pollen *in vitro* from the scanning biological microscope
(A) Normal pollen grains of wild-type plants; (B) control plants; (C) no pollen grains were observed in pTRV2-*pPAP3* silenced plants.

species. *AP3* homology genes are generally expressed in all floral organs (Sasaki et al., 2010; Pan et al., 2011). In the study we identified the *PAP3* gene in pepper. Bioinformatic analysis with putatively orthologous sequences suggests that *PAP3* contains 2 conserved domains and shares a significant sequence similarity to *S. lycopersicum TAP3* (NM_001247148) and *A. thaliana AP3* (NP191002). Expression of *PAP3* in all 4 inner floral organs with abundance in anthers and petals but not in leaves, which was largely consistent with the previous reports of B-function genes. Strong expression of the *PAP3* gene at 4 stages of flower bud development suggests that the *PAP3* gene may be involved in specifying floral organ identities during floral bud differentiation. Higher expression in the restorer line than in the male sterility line indicates that the *PAP3* gene may be involved in the development of pollen in the pepper plant.

AP3 homology genes are originally defined by their capacity to control the development of stamens and petals. In the present study, *PAP3* regulated stamen but not petal development in the pepper plant. One explanation for this phenotype is that the third whorl is more sensitive to B-class genes than the second whole. This model postulates that the third whorl in the development of the stamen requires a greater level of B-class activity than the development of the second petal.

We also applied VIGS to determine the requirement of the *PAP3* gene for stamen and petal identity. The transcripts of *PAP3* in VIGS pepper anthers but not the controls remained at low levels for days. Furthermore, in comparing the morphology of the floral organ, the *PAP3*-silenced anthers appeared shriveled, but petals appeared to develop normally. The results suggest that the *PAP3* gene plays an essential role in the development of pollen in the pepper plant and that silence of *PAP3* gene can lead to male sterility in pepper. To understand the molecular mechanism of CMS in pepper, it would be useful to establish an efficient VIGS technique involving seedling infection to investigate the functions of genes in anther development.

In conclusion, our study is the first report on the cloning and characterization of the MADS-box gene *PAP3* in pepper. Based on a study of its expression pattern and analysis of its function, we suggested that B-function gene *PAP3* may be involved in the development of floral organs and probably plays a crucial role in determining the fertility of pepper. Study of the *PAP3* gene gives us a way of understanding not only the molecular mechanism of CMS in pepper but also the biological functions of MADS-box genes and the role played by the B-function MADS-box gene in the development of the pepper plant's flowers.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (Grant No. 31071806) and Beijing Innovation Consortium of Agriculture Research System (Grant No. BAIC01-2016).

References

Andrews, J., 1995. Peppers: The Domesticated *Capsicums*. University of Texas Press, Austin.

Becker, A., Theissen, G., 2003. The major clades of MADS-box genes and their role in the development and evolution of flowering plants. Mol Phylogenet Evol, 29: 464–489.

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

Chen, C., Liu, S., Hao, X., Chen, G., Cao, B., Chen, Q., Lei, J., 2011. Characterization of a pectin methylesterase gene homolog, *CaPME1*, expressed in anther tissues of *Capsicum annuum* L. Plant Mol Biol Rep, 30: 403–412.

Chung, E., Seong, E., Kim, Y.C., Chung, E.J., Oh, S.K., Lee, S., Park, J.M., Joung, Y.H., Choi, D., 2004. A method of high frequency virus-induced gene silencing in chili pepper (*Capsicum annuum* L. cv. Bukang). Mol Cells, 17: 377–380.

Coen, E.S., Meyerowitz, E.M., 1991. The war of the whorls: Genetic interactions controlling flower development. Nature, 353: 31–37.

Guo, S., Shen, H., Yang, W., Yang, J., Wang, W., 2009a. Isolation of fertility restoration-related ESTs in pepper cytoplasmic male sterility lines using SSH. Acta Horticulturae Sinica, 36: 1443–1449.

Guo, S., Wang, W., Yang, W., Shen, H., 2009b. Quality analysis of subtracted library of fertility restoration-related genes in pepper. Acta Agric Boreali Sin, 24: 161–164.

Hernandez-Hernandez, T., Martínez-Castilla, L.P., Alvarez-Buylla, E.R., 2007. Functional diversification of B MADS-box homeotic regulators of flower development: Adaptive evolution in protein-protein interaction domains after major gene duplication events. Mol Biol Evol, 24: 465–481.

Hileman, L.C., Sundstrom, J.F., Litt, A., Chen, M., Shumba, T., Irish, V.F., 2006. Molecular and phylogenetic analyses of the MADS-box gene family in tomato. Mol Biol Evol, 23: 2245–2258.

Kang, H.G., Jeon, J.S., Lee, S., An, G., 1998. Identification of class B and class C floral organ identity genes from rice plants. Plant Mol Biol, 38: 1021–1029.

Kaufmann, K., Melzer, R., Theiβen, G., 2005. MIKC-type MADS-domain proteins: Structural modularity, protein interactions and network evolution in land plants. Gene, 347: 183–198.

Kim, D.H., Kang, J.G., Kim, B.D., 2007. Isolation and characterization of the cytoplasmic male sterility-associated *orf456* gene of chili pepper (*Capsicum annuum* L.). Plant Mol Biol, 63: 519–532.

Lee, J., Yoon, J., Han, J.H., Lee, W., Do, J., Ryu, H., Kim, S., Park, H., 2010. A codominant SCAR marker linked to the genic male sterility gene (*ms1*) in chili pepper (*Capsicum annuum*). Plant Breed, 129: 35–38.

Leseberg, C.H., Li, A., Kang, H., Duvall, M., Mao, L., 2006. Genome-wide analysis of the MADS-box gene family in *Populus trichocarpa*. Gene, 378: 84–94.

Liu, Y., Schiff, M., Marathe, R., Dinesh-Kumar, S., 2002. Tobacco *Rar1*, *EDS1* and *NPR1/NIM1* like genes are required for N-mediated resistance to *Tobacco mosaic virus*. Plant J, 30: 415–429.

Liu, Y., Nakayama, N., Schiff, M., Litt, A., Irish, V.F., Dinesh-Kumar, S.P., 2004. Virus induced gene silencing of a *DEFICIENS* ortholog in *Nicotiana benthamiana*. Plant Mol Biol, 54: 701–711.

Ma, H., de Pamphilis, C., 2000. The ABCs of floral evolution. Cell, 101: 5–8.

María, J.P., Fernán, F., Consuelo, M., José, T.M., Tania, T., Patricio, A.J., 2007. Isolation of the three grape sub-lineages of B-class MADS-box *TM6*, *PISTILLATA* and *APETALA3* genes which are differentially expressed during flower and fruit development. Gene, 404: 10–24.

Münster, T., Pahnke, J., Di Rosa, A., Kim, J.T., Martin, W., Saedler, H., Theissen, G., 1997. Floral homeotic genes were recruited from homologous MADS-box genes preexisting in the common ancestor of ferns and seed plants. Proc Natl Acad Sci USA, 94: 2415–2420.

Pan, Z.J., Cheng, C.C., Tsai, W.C., Chung, M.C., Chen, W.H., Hu, J.M., Chen, H.H., 2011. The duplicated B-class MADS-box genes display dualistic characters in orchid floral organ identity and growth. Plant Cell Physiol, 52: 1515–1531.

Pnueli, L., Abu-Abeid, M., Zamir, D., Nacken, W., Schwarz-Sommer, Z., Lifschitz, E., 2008. The MADS box gene family in tomato: Temporal expression during floral development, conserved secondary structures and homology with homeotic genes from *Antirrhinum* and *Arabidopsis*. The Plant Journal, 1: 255–266.

Sasaki, K., Aida, R., Yamaguchi, H., Shikata, M., Niki, T., Nishijima, T., Ohtsubo, N., 2010. Functional divergence within class B MADS-box genes *TfGLO* and *TfDEF* in *Torenia fournieri* Lind. Mol Genet Genomics, 284: 399–414.

Shinozuka, Y., Kojima, S., Shomura, A., Ichimura, H., Yano, M., Yamamoto, K., Sasaki, T., 1999. Isolation and characterization of rice MADS box gene homologues and their RFLP mapping. DNA Res, 6: 123–129.

Su, K., Zhao, S., Shan, H., Kong, H., Lu, W., Theissen, G., Chen, Z., Meng, Z., 2008. The MIK region rather than the C-terminal domain of AP3-like class B floral homeotic proteins determines functional specificity in the development and evolution of petals. New Phytol, 178: 544–558.

Theissen, G., Becker, A., Di Rosa, A., Kanno, A., Kim, J.T., Munster, T., Winter, K.U., Saedler, H., 2000. A short history of MADS-box genes in plants. Plant Mol Biol, 42: 115–149.

Whipple, C.J., Ciceri, P., Padilla, C.M., Ambrose, B.A., Bandong, S.L., Schmidt, R.J., 2004. Conservation of B-class floral homeotic gene function between maize and *Arabidopsis*. Development, 131: 6083–6091.

Willcox, M.C., Reed, S.M., Burns, J.A., Wynne, J., 1991. Effect of microspore stage and media on anther culture of peanut (*Arachis hypogaea* L.). Plant Cell Tissue Organ Cult, 24: 25–28.