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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

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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

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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.