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Virus-induced gene silencing of *PEAM4* affects floral morphology by altering the expression pattern of *PsSOC1a* and *PsPVP* in pea

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ABSTRACT

pea-MADS4 (*PEAM4*) regulates floral morphology in *Pisum sativum* L., however, its molecular mechanisms still remain unclear. Virus-induced gene silencing (VIGS) is a recently developed reverse genetic approach that facilities an easier and more rapid study of gene functions. In this study, the *PEAM4* gene was effectively silenced by VIGS using a pea early browning virus (PEBV) in wild type pea *JI992*. The infected plants showed abnormal phenotypes, as the floral organs, especially the sepals and petals changed in both size and shape, which made the corolla less closed. The petals changed in morphology and internal symmetry with, the stamens reduced and carpel dehisced. Larger sepals and longer tendrils with small cauline leaves appeared, with some sepals turning into bracts, and secondary inflorescences with fused floral organs were formed, indicating a flower-to-inflorescence change. The infected plants also displayed a delayed and prolonged flowering time. The PEAM4-VIGS plants with altered floral morphology were similar to the *pim* (*proliferating inflorescence meristem*) mutant and also mimicked the phenotypes of *ap1* mutants in *Arabidopsis*. The expression pattern of the homologous genes *PsSOC1a* and *PsSVP*, which were involved in flowering time and florescence morphological control downstream of *PEAM4*, were analyzed by real-time RT-PCR and mRNA *in situ* hybridization. *PsSOC1a* and *PsSVP* were ectopically expressed and enhanced in the floral meristems from *PEAM4*-silenced plants. Our data suggests that *PEAM4* may have a similar molecular mechanism as *AtAP1*, which inhibits the expression of *PsSOC1a* and *PsSVP* in the floral meristem from the early stages of flower development. As such, in this way *PEAM4* plays a crucial role in maintaining floral organ identity and flower development in pea.

Introduction

Pea (*Pisum sativum* L.) is one of the most important agricultural products in the world and also one of the most classic materials for genetic research. Many studies have been done on pea flower development in order to investigate methods that increase production. Several genes involved in flower development in pea have been cloned and studied (Hecht et al., 2005). Studies on the legume, however, is far behind the relative work done on rice and *Arabidopsis* because of the lack of large genome sequences and lack of an effective genetic transformation method.

Virus-induced gene silencing (VIGS) is a recently available method to study gene functions in pea using vectors reconstructed from PEBV (pea early browning virus) and the binary plasmid of pCAMBIA1300 (Constantin et al., 2004). We improved the method and effectively silenced *PsPI* gene in the wild type pea *JI992*. Further, we used VIGS to successfully silence the whole gene family of *PsAGs* by generating vectors targeting the most conserved regions and analyzed their biological functions (data not shown). This improved VIGS method in pea offers an opportunity for use with partially mimic loss-of-function mutants, which previously were not easily obtained.

PEAM4 (*pea-MADS4*) from *P. sativum* L. is a homologous gene of *AP1* (*APETALA1*) in *Arabidopsis thaliana* with 76% similarity in the amino-acid sequence between their coding proteins (Berbel et al., 2001). They share the same expression pattern, which is first detected in the emerged inflorescence primordia and become dispersed after emerging (Mandel et al., 1992; Berbel et al., 2001). Within the development of the flower, *PEAM4* could only be detected in sepals and petals, but not in stamens and carpel, indicating functions as A type genes in the ABC model (Coen and Meyerowitz, 1991; Berbel et al., 2001). Plants with mutations in the *PEAM4* genes were obtained as *proliferating inflorescence meristem* (*pim*). Delayed floral meristem specification and altered floral morphology were observed in *pim* mutants. In place of floral meristems, additional secondary-like inflorescences were produced and each of these inflorescences bore two or more abnormal flowers. The floral morphology of *pim* mutants was aberrant in that first-whorl

Abbreviations: EV, empty vector; PEBV, pea early browning virus; *pim*, *proliferating inflorescence meristem*; PTGS, post-transcriptional gene silencing; VIGS, virus-induced gene silencing.

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sepals were replaced by leafy bract-like structures, and second- and third-whorl organs were either absent or mosaic (Taylor et al., 2002). A similar phenotype was reported in the *ap1* mutant in *Arabidopsis*, which might be caused by an altered expression of a group of flower-related genes, such as *AtSOC1* (*SUPPRESSOR OF OVEREXPRESSION OF CO1*), *AtSVP* (*SHORT VEGETATIVE PHASE*) and *AtAGL24* (*AGAMOUS-LIKE24*) (Irish and Sussex, 1990; Liu et al., 2007; Kaufmann et al., 2010; Lee and Lee, 2010). These genes interacted with *AtAP1* and inhibited the expression of downstream genes at the early stage of flower development (Gregis et al., 2009; Liu et al., 2009). *AtSOC1*, *AtSVP*, and *AtAGL24* blocked the expression of *AtSEP3* (*SEPALLATA3*) through different pathways (Gregis et al., 2009). *AtAP1* down-regulated expression as the flower developed (Wellmer et al., 2006; Liu et al., 2007; Kaufmann et al., 2010), and finally triggered the expression of *AtSEP3*, which led to further floral morphogenesis in an *AtAP1* dependent way (Gregis et al., 2008, 2009; Liu et al., 2009; Kaufmann et al., 2010).

For the purpose of clarifying whether there are similar regulation pathways in a model legume, and especially the possible functions of *PEAM4*, we generated PEAM4-VIGS vectors with the aim to obtain *PEAM4* silenced plants based on our improved method. Furthermore, we intended to illustrate the possible function of *PEAM4* and the associated regulation pathways in maintaining floral morphology by analyzing phenotype alteration and detecting the expression change of *PsSVP* and *PsSOC1a* in *PEAM4-VIGS* plants.

Materials and methods

Plant material and growth conditions

Wild type pea *JI992* was kindly provided by Mike Ambrose (John Innes Centre, Norwich, UK). Seeds were surface sterilized and soaked in distilled water for 24 h. After washing three times, seeds were potted in soil and then transferred to a growth chamber at 20 °C under a 16-h-light/8-h-dark photoperiod.

Constructs and generation of VIGS plants

The vectors used in the virus-induced gene silencing included pCAPE1, pCAPE2-GFP, and pCAPE2-PDS that were kindly provided by Ida Elisabeth Johansen (Department of Plant Biology, Danish Institute of Agricultural Sciences, Frederiksberg, Denmark). pCAPE1 contained full-length cDNA of PEBV RNA-1 with an intron inserted to stabilize the plasmid in bacteria. pCAPE2-GFP contained the cDNA of PEBV RNA-2 with the *GFP* coding sequence used for control as an empty vector (EV control) (Fig. 1A–B). pCAPE2-PDS had a 470 bp cDNA fragment of *Pisum sativum* phytoene desaturase (PDS) inserted in the RNA-2 cDNA. To generate the *PEAM4-VIGS* vectors, a 505-bp fragment including the gene specific region of *PEAM4* (GeneBank: *AJ279089.1*) was amplified using cDNAs from the shoot apical meristem. The amplified fragments were double digested by *XbaI*/*PstI* to substitute the PDS fragment downstream of the CaMV (Cauliflower mosaic virus) 35S promoter in pCAPE2-PDS vector to obtain pCAPE2-*PEAM4* (Fig. 1C and D). Both pCAPE2-*GFP* and pCAPE2-*PEAM4* constructs were transformed into *Agrobacterium tumefaciens GV3101* by heat shock, and were used to transform pea leaves by the syringe infiltration method (Constantin et al., 2004; Gronlund et al., 2010). Each inoculation was carried out three times and each time more than eight different seedlings were infiltrated. All primers used in this work are listed in Table 1. The clones used for vector construction were verified by sequencing.

Gene expression analysis

Total RNA was isolated from small floral buds of early plants using TRIzol reagent (Invitrogen, Shanghai, China), and treated with RNase-free DNaseI (TakaRa, Dalian, China). Treated RNA (2 μg) was used for the first-strand cDNA synthesis using a PrimeScript RT Reagent Kit (TakaRa, Dalian, China). Real-time PCR was performed with Applied Biosystems StepOne™ and a StepOnePlus™ System with the SYBR Premix Ex Taq (Perfect Real Time) Kit (TakaRa, Dalian, China). The primers of *PEAM4*, *PsSOC1a* (GeneBank: *AY830920*) and *PsSVP* (GeneBank: *AY830919*) were designed using Primer Premier 5, and relative transcript levels were normalized using *PsEF1α* (*Elongation Factor 1 alpha*) as a standard. Data were analyzed wirh StepOne Software v2.1. The primers used for qRT-PCR are also described in Table 1.

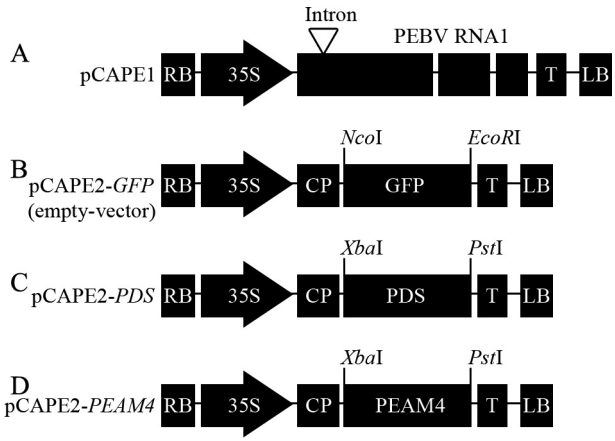

Fig. 1. Construction of VIGS vectors. Pea early browning virus (PEBV) expression cassettes of RNA-1 and RNA-2 were inserted between the right and left border (RB and LB) of a pCAMBIA1300 derived plasmid. Transcriptional control was exerted by a 35S promoter and a NOS terminator (T). (A) pCAPE1 containing full-length cDNA of PEBV RNA-1 with an intron inserted to stabilize the plasmid in bacteria. (B) pCAPE2-GFP containing cDNA of PEBV RNA-2 with a GFP coding sequence replaced the genes required for nematode transmission. CP is the coat protein coding region. (C) pCAPE2-PDS with a 470 bp cDNA fragment of *Pisum sativum* phytoene desaturase (PDS) inserted in the RNA-2 cDNA. (D) pCAPE2-PEAM4 containing PEAM4 cDNA inserted in the RNA-2 cDNA. The restriction sites displayed are not found elsewhere in the pCAPE2 vector and can be used for insertion of other gene fragments.

RNA in situ hybridization

RNA *in situ* hybridization with digoxigenin-labelled probes was performed on 8 μm longitudinal paraffin sections of pea floral meristems as described (Brewer et al., 2006). RNA antisense and sense probes were generated from 370 bp of *PsSVP* and 443 bp of *PsSOC1a* cDNAs, respectively, and cloned into the pTA2-vector

Table 1
List of primers used in this study.

Primer name	Sequence (5′–3′)
vPEAM4F	TCTAGATATGCCACTGATTCTTGC (*Xba*I)
vPEAM4R	CTGCAGCCATTTCTGGTGCTTCCT (*Pst*I)
qPEAM4F	AGCTTGACTTGACCCTTGAACCAC
qPEAM4R	TCATAACACCGACCACCTCCCAAA
qSVPF	CTCTTGAAATTGGCTTGGGTCGTG
qSVPR	TCATGCCCGTCACATGTCGCTTTA
qSOC1aF	CATGCAGGACACGATTGAACGGTA
qSOC1aR	CCATGAGTTTCCGTTTCGAGGCTT
qEF1αF	GATGCACCTGGACATCGTGAC
qEF1αR	CTTAGGGGTGGTAGCATCCATCT
ishPEAM4F	TCGTACACGCAGAAACCAACTC
ishPEAM4R	ATAACACCGACCACCTCCCAA
ishSVPF	CCGTTCTCTGTGATGCTG
ishSVPR	TGTCTTCCCTTTCTTTGG
ishSOC1aF	GTAGAAATACCAGAAGTGC
ishSOC1aR	TCAACTAGACCTGTGTAGG

v for VIGS; q for quantification; ish for *in situ* hybridization; F for forward; R for reverse. The underlined nucleotides refer to restriction sites in the parentheses.