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The *CRC* orthologue from *Pisum sativum* shows conserved functions in carpel morphogenesis and vascular development

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• **Background and Aims** *CRABS CLAW* (*CRC*) is a member of the YABBY family of transcription factors involved in carpel morphogenesis, floral determinacy and nectary specification in arabidopsis. *CRC* orthologues have been functionally characterized across angiosperms, revealing additional roles in leaf vascular development and carpel identity specification in Poaceae. These studies support an ancestral role of *CRC* orthologues in carpel development, while roles in vascular development and nectary specification appear to be derived. This study aimed to expand research on *CRC* functional conservation to the legume family in order to better understand the evolutionary history of *CRC* orthologues in angiosperms.
• **Methods** *CRC* orthologues from *Pisum sativum* and *Medicago truncatula* were identified. RNA i*n situ* hybridization experiments determined the corresponding expression patterns throughout flower development. The phenotypic effects of reduced *CRC* activity were investigated in *P. sativum* using virus-induced gene silencing.
• **Key Results** *CRC* orthologues from *P. sativum* and *M. truncatula* showed similar expression patterns, mainly restricted to carpels and nectaries. However, these expression patterns differed from those of other core eudicots, most importantly in a lack of abaxial expression in the carpel and in atypical expression associated with the medial vein of the ovary. *CRC* downregulation in pea caused defects in carpel fusion and style/stigma development, both typically associated with CRC function in eudicots, but also affected vascular development in the carpel.
• **Conclusions** The data support the conserved roles of *CRC* orthologues in carpel fusion, style/stigma development and nectary development. In addition, an intriguing new aspect of CRC function in legumes was the unexpected role in vascular development, which could be shared by other species from widely diverged clades within the angiosperms, suggesting that this role could be ancestral rather than derived, as so far generally accepted.

Key words: Flower development, *CRABS CLAW*, *CRC* orthologues, carpel evolution, gynoecium, nectary, carpel vasculature, floral determinacy, *Pisum sativum*, *Medicago truncatula*, YABBY transcription factors.

INTRODUCTION

Carpels are the female reproductive organs of the angiosperm flower that enclose the ovules, which thus do not remain naked or exposed, as they do in gymnosperm species. Carpels may develop as separate structures or may be fused into a syncarpic pistil, typically comprising a set of characteristic tissues specialized for different roles. Thus, stigmatic cells at the apical end of the pistil capture and germinate the pollen grains; these produce pollen tubes that are transmitted through the style to reach the basal ovary, a chamber that contains the ovules and where fertilization takes place. Then, the fertilized pistil becomes a fruit, an organ that ensures protection through seed development and later provides a seed dispersal mechanism. These specialized functions have great adaptive value, and carpels and fruits are generally acknowledged as major factors for the huge evolutionary success of angiosperms among extant plants (Scutt *et al*., 2006; Ferrandiz *et al*., 2010). The study of the origin of the carpel and how morphological innovations have shaped this organ are thus central questions for plant evolutionary biology. In this context, the functional characterization of genes potentially involved in the origin of the carpel, including comparative studies across several angiosperm clades, constitutes a major field of evo-devo research.

CRABS CLAW (*CRC*) belongs to the YABBY family of transcription factors, which in *Arabidopsis thaliana* consists of six members (Bowman and Smyth, 1999; Siegfried *et al*., 1999). *YABBY* genes are specific to seed plants, but the *CRC* subfamily forms a single orthologous lineage restricted to angiosperms, as deduced from phylogenetic analyses and strongly supported by the recent publication of the *Amborella trichopoda* genome sequence (Lee *et al*., 2005; Yamada *et al*., 2011; Bartholmes *et al*., 2012; Amborella Genome Project, 2013). In arabidopsis, *AtCRC* has been shown to participate in nectary specification and, more importantly, in the development of specific characters that define the angiosperm carpel, such as the differentiation of the stigma and the style, the fusion of carpel margins, which allows ovule enclosure, and the determination of the floral meristem (Alvarez and Smyth, 1999; Bowman and Smyth, 1999). These functions, together with the angiosperm-specific nature of the CRC clade, have led to the proposal that *CRC* genes are putative factors whose neofunctionalization may have been essential for the evolutionary origin of the carpel, prompting a wealth of comparative studies on *CRC* orthologues across angiosperms,

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including monocots, basal dicots and eudicots. For example, in *Oryza sativa* (rice), the *CRC* orthologue *DROOPING LEAF* (*DL*) is required for floral meristem determinacy and carpel organ identity (Nagasawa *et al*., 2003; Yamaguchi *et al*., 2004); it also has extra-floral functions, such as an essential role in leaf midrib formation, functions shared by *CRC* orthologues from other monocot species (Yamaguchi *et al*., 2004; Ishikawa *et al*., 2009; Wang *et al*., 2009; Nakayama *et al*., 2010). In basal eudicots, such as *Eschscholzia californica*, it was shown by virus-induced gene silencing (VIGS) that the *EcCRC* gene controlled meristem determinacy, carpel polarity and ovule initiation (Orashakova *et al*., 2009). Functional studies have also been carried out in Solanaceae, such as *Petunia hybrida* and *Nicotiana tabacum*, uncovering roles similar to those described for arabidopsis, including nectary specification (Lee *et al*., 2005). In addition, expression analyses of *CRC*-like genes are available for several species of other basal eudicots, such as *Aquilegia formosa* and the basal angiosperm *Amborella trichopoda* (Fourquin *et al*., 2005; Lee *et al*., 2005).

These comparative studies support an ancestral role of *CRC* genes in the specification of carpel polarity, floral meristem termination and probably stigma and style formation (Yamada *et al*., 2011), while *CRC* function in nectary development appears to be the consequence of *CRC* neofunctionalization in core eudicots, and a similar scenario is proposed for the independently acquired role of *CRC* genes in midrib development in grasses (Lee *et al*., 2005; Nakayama *et al*., 2010; Yamada *et al*., 2011).

Here, we aimed to expand the comparative studies on *CRC*-like genes to legumes, a large family of plants of great economic and ecological importance belonging to the rosid clade within the core eudicots. We have used recently developed tools that allow functional characterization of developmental genes, namely VIGS protocols optimized for *Pisum sativum* (Constantin *et al*., 2004). Our work highlights the conservation in legume species of the major roles described for *CRC* orthologues in other eudicots, while also uncovering an unexpected function of legume *CRC* genes in vascular development that questions the generally accepted view of the derived functions of *CRC* orthologues in monocot species.

MATERIALS AND METHODS

Plant material and growth conditions

Pisum sativum and *Medicago truncatula* plants were grown in the greenhouse at 22 °C (day) and 18 °C (night) with a 16-h light/8-h dark photoperiod, in soil irrigated with Hoagland no. 1 solution supplemented with oligoelements (Hewitt, 1966).

Cloning and sequence analysis

A BLAST search identified an *M. truncatula* coding sequence of a putative *CRC* orthologue (accession number XM_003614153). We named the corresponding coding sequence *MtCRC*. The full-length coding sequence of the *P. sativum CRC* gene was isolated by RT-PCR and TAIL PCR on cDNA of young flowers of *P. sativum*. We first used the degenerate primers CRCFordeg (TTGGACACAGTGACAGTGAAGTGYGGNCA YTG) and CRCRevdeg (AGCCCAATTCTTAGCAGCAGC ASWRAANGCYTC), designed from the conserved zinc finger and YABBY domains of *MtCRC*, to isolate a partial coding sequence of *PsCRC*. We then used the primers PsCRCFor2 (CTCTTTTCTCACAACAAGACC) and RT (AACTGGAAGA ATTCGCGGCCGCAGGAAT; sequence added to the oligodT primer used for retrotranscription) to obtain the 3′ end of *PsCRC*. Finally, TAIL-PCR (thermal asymmetric interlaced PCR) reactions allowed us to obtain the 5′ end of *PsCRC* using random nested oligos (Fourquin and Ferrandiz, 2014) and the specific nested oligos PsCRCTAIL1 (GACAACAAATGGTG GTGGTTTAG), PsCRCTAIL2 (GAAGAGGAAGACGATGG TTGTC) and PsCRCTAIL3 (GAATTTGGTGGTCTTGTTG TGAG). The full-length *PsCRC* coding sequence has been deposited in GenBank under the accession number KF806036. The deduced amino acid sequence alignments were analysed using Macvector 12·6 software. A neighbour-joining tree was estimated from distance matrices from 10 000 bootstrap replicates.

In situ *hybridization*

RNA *in situ* hybridization with digoxigenin-labelled probes was performed on 8-µm paraffin sections of *M. truncatula* and *P. sativum* buds as described by Ferrándiz *et al*. (2000). The RNA antisense and sense probes were generated from a 268-bp fragment of the *PsCRC* cDNA (positions 190–457) and *MtCRC* from a 262-bp fragment (positions 162–423). Both fragments were cloned into the pGemT-Easy vector (Promega), and sense and antisense probes were synthesized using the corresponding SP6 or T7 polymerases.

Virus-induced gene silencing in P. sativum

The same region of *PsCRC* coding sequence as that used for *in situ* hybridization was used for the VIGS experiments. An Xba1 restriction site was added to the 5′ end of the PCR fragment and an EcoRI restriction site was added to the 3′ end. The amplicon was digested with Xba1 and EcoRI and cloned into a similarly digested pCAPE2 vector. The resulting plasmid, pCAPE2-PsCRC, was confirmed by digestion and sequencing, before introduction into *Agrobacterium tumefaciens* strain GV3101. In addition, the empty vector pCAPE2 was used as a negative control and the pCAPE2-PsPDS, possessing 400 pb of the *P. sativum PHYTOENE DESATURASE* gene, was used as a positive control. Agroinoculation of *P. sativum* leaves was performed as described (Constantin *et al*., 2004). The VIGS experiments were performed twice and similar results were obtained in both analyses.

Quantitative RT-PCR

Total RNA was extracted from flowers in anthesis with the RNeasy Plant Mini kit (Qiagen). Four micrograms of total RNA was used for cDNA synthesis performed with the First-Strand cDNA Synthesis kit (Invitrogen) and the qPCR master mix was prepared using iQTM SYBR Green Supermix (Bio-Rad). The primers used to amplify *PsCRC* (qPsCRCFor, TTCATCCCTAATTCACCAACCAG; qPsCRCRev, ATCCT CATTGATGCCATAAAACTTT) generated products of 51 bp. Results were normalized to the expression of the *ACTIN* reference as previously described (Weller *et al*., 2009). The

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efficiencies of amplification of *PsCRC* and the reference gene were similar. The PCR reactions were run and analysed using the ABI PRISM 7700 Sequence Detection System (Applied Biosystems).

Histology

Pistils were collected for histological analyses, fixed in FAE (3·7 % formaldehyde, 5 % acetic acid, 50 % ethanol, v/v) under vacuum and then embedded in paraffin. Sections of 10-μm were stained in 0·2 % toluidine blue solution and observed under a microscope (Nikon Eclipse E-600). For detailed analyses of vascular strands, tissue was embedded in hydroxyethyl methacrylate (Technovit 7100) according to the protocol of the manufacturer (KulzerHisto-tec, Wehrheim, Germany). After embedding, the material was placed in a mould, and 1 mL of Hardener II was added per 15 mL of Technovit, resulting in a polymerization reaction. The Technovit blocks were sectioned with a Reichert Jung Ultracut E microtome at 3 μm and stained in 0·1 % toluidine blue.

RESULTS

Identification of CRC *homologues in* M. truncatula *and* P. sativum

In order to identify homologues of *CRC* in *M. truncatula* and *P. sativum*, we first performed a BLAST search against public databases. This search identified the *M. truncatula CRC* complete cDNA sequence (accession number XM_003614153) and a corresponding genomic sequence (accession number AC117656·24), referred to as *MtCRC* in this study. As the BLAST search did not render any result for *P. sativum* we designed degenerate primers based on the two YABBY conserved domains. One putative *CRC* orthologue, named *PsCRC*, was amplified from cDNA of young flowers of *P. sativum*. The complete coding sequence was subsequently obtained by TAIL-PCR and the use of an adapted oligodT primer. The predicted MtCRC and PsCRC proteins possessed the typical zinc finger and YABBY domains shared by all members of the family as well as CRC-specific motifs (Fig. 1A; Bartholmes *et al*., 2012). Figure 1B shows a neighbour-joining tree constructed by comparing the full-length deduced protein sequences of MtCRC, PsCRC and several CRC orthologues from different species, and also including the sequences of other arabidopsis YABBY factors. This analysis showed that both MtCRC and PsCRC are most closely related to core eudicot CRC proteins (Fig. 1B).

The CRC *genes from legumes are expressed in carpels*

Medicago truncatula and *P. sativum* have flowers of similar morphology. They are composed of five sepals, five petals, ten stamens and one carpel, arranged in four whorls. The five sepals are fused at their base and the corolla contains three different types of petals: the large standard at adaxial position, the wings at lateral positions and two smaller petals fused at the abaxial position (Fig. 2A, B). In the third whorl, nine stamens are fused, forming a long tubular structure, whereas a tenth, free stamen is placed at the adaxial side of the flower. The staminal tube surrounds a single carpel whose margins are fused at the adaxial side of the flower and which possesses two lines of ovules developing on each side of the fused carpel margins (Ferrándiz *et al*., 1999; Benlloch *et al*., 2003). Between the third and fourth whorls, an inconspicuous nectary gland develops at the base of the staminal tube (Teuber *et al*., 1980; Razem and Davis, 1999). Floral ontogeny in *P. sativum* and *M. truncatula* has been described in detail, showing a characteristic early development of the carpel primordium that precedes stamen primordium differentiation (Ferrándiz *et al*., 1999; Benlloch *et al*., 2003).

To get a detailed expression pattern of legume *CRC* orthologues during flower development, we performed *in situ* hybridization on flowers from early stages of development until anthesis. In young floral buds, *PsCRC* could be detected uniformly throughout the carpel (Fig. 2C). Later in development, *PsCRC* expression disappeared from most of the ovary wall, becoming restricted to a specific area around the medial vascular vein (Fig. 2D, E). In stage 7 flowers (as defined in Ferrándiz *et al*., 1999), when the style has not yet differentiated, *PsCRC* was strongly expressed in a ring of cells of the carpel opposite to the fused carpel margins, and therefore positioned in the abaxial side of the flower, surrounding the domain where the medial vein of the carpel will develop (Fig. 2D). The same pattern was observed in older stages when the carpel apical regions had already differentiated (Fig. 2E). At anthesis, *PsCRC* expression was still detected associated with the medial vein of the carpel wall, but instead of in a ring shape, its expression was restricted to a small patch of cells between the vasculature and the ovary chamber (Fig. 2F). *PsCRC* transcripts were present at this position all along the ovary wall (Fig. 2I) and strongly in the style–stigma junction and in stigmatic tissues (Fig. 2G, H). In addition, *PsCRC* expression was very high in nectaries, also expanding to the base of the staminal tube in young flowers (Fig. 2E). This result was confirmed by qRT-PCR on leaves and different flower organs of anthesis flowers: *PsCRC* was absent from leaves and perianth organs, weakly present in stamens and strongly expressed in carpels (Fig. 2L). A similar expression pattern was observed for the *CRC* orthologue of *M. truncatula. MtCRC* expression was detected in a ring of cells around the carpel medial vasculature from early stages of development until anthesis (Fig. 2J, K). *MtCRC* was also detected, like *PsCRC*, at the base of the developing staminal tube, where the nectary develops (Fig. 2 K). Like *PsCRC* expression, *MtCRC* expression was not found in leaves or any other vegetative organ according to the Mt Map Expression Atlas (http://mtgea.noble.org/v3/; Benedito *et al*., 2008) (Fig. 2M)

PsCRC *controls carpel fusion and style development*

To investigate the function of *PsCRC* in *P. sativum*, we used VIGS to reduce its transcript levels. This method results in transitory downregulation of a specific gene via modified plant viruses and it has been shown to efficiently direct the degradation of endogenous mRNAs in several species (Ratcliff *et al*., 2001; Hileman *et al*., 2005; Wege *et al*., 2007), including *P. sativum*, for which a specific protocol has been successfully set up (Constantin *et al*., 2004). Fifty plants of *P. sativum* 'Bonneville' were infiltrated with the pCAPE2-PsCRC construct, ten with the pCAPE2-PsPDS, which targets the *P. sativum PHYTOENE DESATURASE* (*PDS*) gene, as positive control, and ten with the empty vector as negative control. All the plants infiltrated with the pCAPE2-PsPDS construct showed

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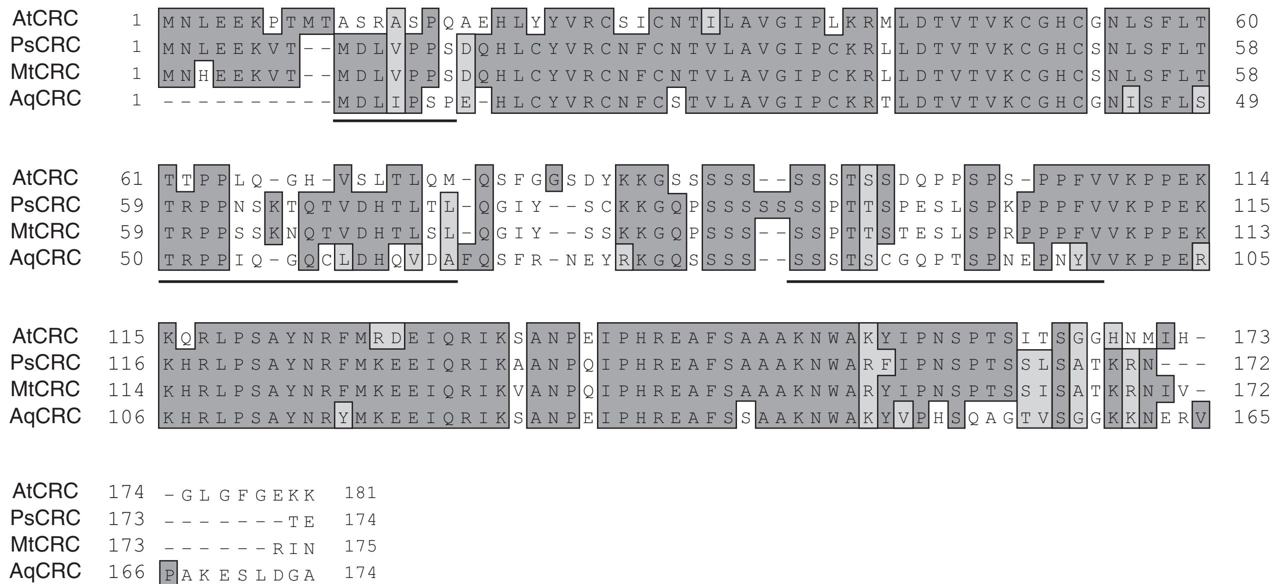

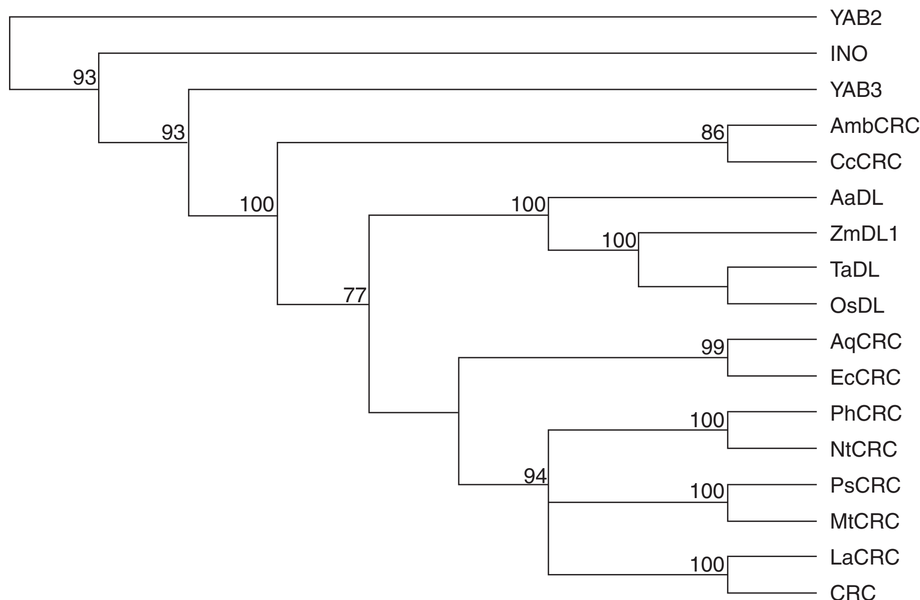

FIG. 1. Sequence analyses of CRC homologues. (A) Multiple sequence alignment of deduced polypeptides of *Arabidopsis thaliana* AtCRC (NM_105585), PsCRC, MtCRC and *Aquilegia formosa* AqCRC (AY854797). Specific CRC motifs according to Bartholmes *et al.* (2012) are underlined. (B) Neighbour-joining tree of YABBY deduced polypeptides from selected species. *Cabomba caroliniana* CcCRC (AB553318), *Amborella trichopoda* AmbCRC (AJ877257), *Oryza sativa DROOPING LEAF* OsDL (AY494713), *Triticum aestivum* TaDL (AB470269), *Zea mays* ZmDL1 (NM_001155258), *Asparagus asparagoides* AaDL (AB535099), *Aquilegia formosa* AqCRC (AY854797), *Eschscholzia californica* EcCRC (AM946412), *Nicotiana tabacum* NtCRC (AY854799), *Petunia hybrida* PhCRC (AY854801), *Lepidium africanum* LaCRC (AY854802), *Arabidopsis thaliana* CRC (NM_105585), MtCRC and PsCRC. The arabidopsis *YABBY* genes *YAB2* (AAD33716), *YAB3* (AF136540) and *INNER NO OUTER* (*INO*) (NM_102191) were included in the analyses. Numbers on branches indicate bootstrap values for 10 000 replicates. *YAB2* was used to root the tree.

substantial zones of white tissue in every developing organ, indicating the success of the downregulation process. In contrast, the negative control plants did not show any developmental phenotypes compared with the wild-type plants. To evaluate the efficiency of the VIGS treatment we also measured by qRT-PCR the level of expression of *PsCRC* on flowers from four different treated plants showing altered phenotypes. In all cases expression of *PsCRC* was reduced to 11–52 % of wild-type level (Fig. 3I), indicating that the VIGS treatment was highly effective. The first five flowers were observed at anthesis for each

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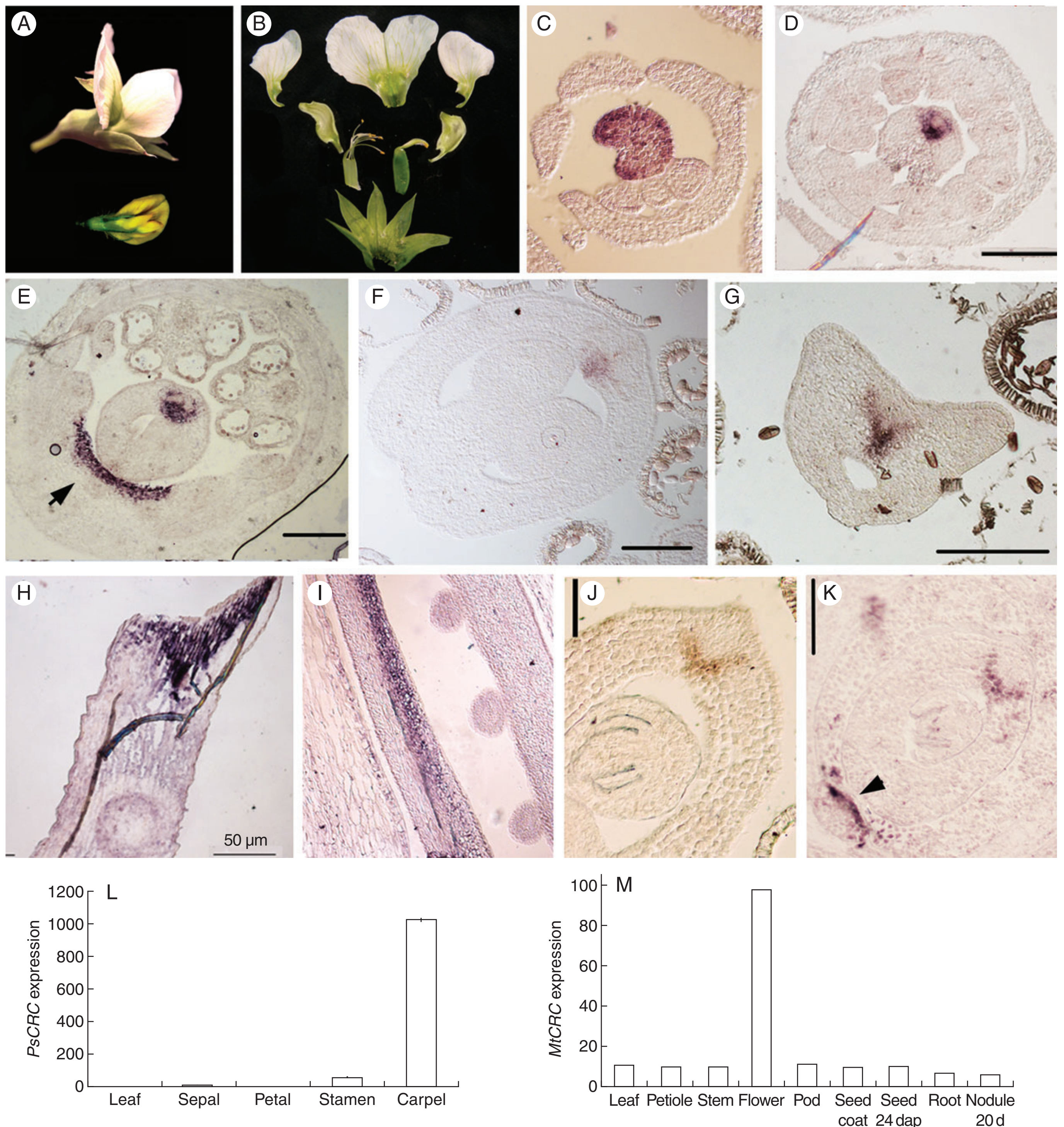

FIG. 2. Expression analyses of homologues of *CRC* in *P. sativum* and *M. truncatula* flowers. (A) Anthesis flowers of *P. sativum* (top) and *M. truncatula* (bottom). (B) Dissected floral organs from a pea flower at anthesis. (C–I) *Pisum sativum* flowers probed with *PsCRC*: transverse sections (C–G) and longitudinal sections (H, I). (C) *PsCRC* transcripts accumulate uniformly in young carpel primordia at stage 6. (D–G) Later in development, *PsCRC* transcripts become associated with the main vascular vein in the carpel wall. (D) In a stage 7 flower, *PsCRC* expression is detected as a ring surrounding the vein. (E) At stage 8, the expression pattern is similar. Strong expression is also detected in the area of the staminal tube where the nectary develops (arrow). (F) In the anthesis carpel, *PsCRC* is detected adaxially to the vein. (G) Strong expression is also detected in a transverse section at the ovary–style junction of an anthesis carpel. (H) Longitudinal section of an anthesis carpel showing strong *PsCRC* expression at the style and stigma. (I) Longitudinal section of the ovary of an anthesis carpel, where *PsCRC* transcripts can be detected adaxially to the vein. (J, K) Transverse sections of *M. truncatula* stage 8 flowers probed with *MtCRC*. *MtCRC* expression is visible in the carpel in a ring-shaped pattern around the vasculature (J) and in the area where nectaries develop at the base of the staminal tube (arrowhead in K). (L) Expression levels of *PsCRC* in different organs of *P. sativum* flowers at anthesis, determined by qRT-PCR. (M) Expression levels of *MtCRC* in different organs. Values correspond to the level of Mtr.22428·1.S1_at Affymetrix probe signal from the Medicago Gene Expression Atlas (Benedito *et al*., 2008). *Pisum sativum* developmental stages from Ferrandiz *et al*. (1999). dap, days after planting. *Medicago truncatula* stages according to Benlloch *et al*. (2003). Scale bar = 100 µm except in (H, J) = 50 µm.

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PsCRC-VIGS-inoculated plant (250 flowers in total). In 37 % of the cases, flowers showed abnormal carpel development, with ovaries reduced in length (Fig. 3). All the affected carpels showed fusion defects, displaying an aperture in the upper half of the ovary ranging from 1 to 2 mm in the less affected flowers to almost 1 cm in the most severe cases (Fig. 3B–E). The disruptions of carpel closure led to the development of external ovules, ranging in number from one to four. Apical carpel tissue development was also affected in the PsCRC-VIGS-inoculated plants. In the wild-type carpel, the style emerges almost perpendicularly to the ovary, ending in a hairy stigmatic zone in its adaxial side (Fig. 3F). In PsCRC-VIGS carpels, styles were shorter and grew at a wider angle compared with the wild-type (Fig. 3G, H). In the most severely affected carpels, styles were narrow and short, and grew almost parallel to the longitudinal axis of the ovary. In addition, stigmatic tissue was strongly reduced (Fig. 3C, D, H). Seed set in PsCRC-VIGS pods was reduced to one or two seeds in the most affected lines compared with three to five seeds in wild-type plants. Histological sections of PsCRC-VIGS carpels further confirmed the defects in stigma, style and ovary development (Fig. 4A–H). Defects of carpel closure were visible from the style to more basal positions in the ovary, where ovules developed outwardly at the unfused carpel margins (Fig. 4E, H). In addition, developmental defects were also visible in the ovary medial vascular veins: in PsCRC-VIGS carpels, medial vascular bundles showed clear morphological differences when compared with wild-type (Fig. 5). The overall shape of a transverse section of the vein in wild-type carpels is shown in Fig. 5A, B: xylem/ protoxylem cells are positioned adaxially; phloem cells, which appear densely stained, form a band of tissue adjacent to xylem, and are abaxially capped by a few cell layers of fibre cells (Fig. 5C, F); and the whole bundle has a lenticular/pyramidal shape typical of collateral veins. In PsCRC-VIGS carpels, the overall transverse shape of the vein was circular (Fig. 5G, H); phloem cell number appeared strongly reduced, while adaxial xylem and protoxylem were well developed; abaxially, fibre cell layers appeared increased in number, and protoxylem/xylem cells were also found adjacent to phloem, visible as large empty cells with thickened walls. Overall, these phenotypes were consistent with a slight adaxialization of the bundle in the PsCRC-VIGS carpels (Fig. 5I, L).

DISCUSSION

CRC *expression in legumes does not follow the most common pattern in other eudicots*

The legume *CRC* orthologues studied in this work, *PsCRC* and *MtCRC*, show very similar expression domains, suggesting that this might be a general pattern for at least legumes in the inverted repeat-lacking clade (IRLC), to which both species belong (Lavin *et al.*, 2005). Like other *CRC* orthologues from eudicot species, legume *CRC* genes are strongly expressed in nectaries and in carpels. In carpels, however, expression is initiated uniformly throughout the pistil primordia and later it is found associated with the medial vein and in the apical tissues, a pattern that differs significantly from those described for *CRC* orthologues in other eudicot species. For example, in arabidopsis, *AtCRC* is expressed in the abaxial side of the lateral domains of young pistil primordia, while at later developmental stages it is restricted to the epidermis of the valves, internally associated with placentae, and also strongly expressed in the apical domain that will differentiate into style and stigma (Bowman and Smyth, 1999; Lee *et al.*, 2005). The expression of *CRC* in abaxial carpel domains and apical carpel tissues has been described in several other eudicot species, such as *P. hybrida* (Lee *et al.*, 2005) and *E. californica* (Orashakova *et al.*, 2009), in the monocot *Asparagus asparagoides* (Nakayama *et al.*, 2010) and in basal angiosperms such as *Amborella trichopoda* (Fourquin *et al.*, 2005) and *Cabomba caroliniana* (Yamada *et al.*, 2011), and therefore is generally considered to be ancestral. Legume *CRC* genes lack abaxial-specific expression, as has also been described in monocots of the Poaceae family, in which *CRC* genes are expressed uniformly throughout the carpel primordia (Yamaguchi *et al.*, 2004; Ishikawa *et al.*, 2009). Given the evolutionary distance between legumes and grasses, it is likely that the independent loss of regulatory elements may have resulted in these similar patterns in both clades.

On the other hand, legume *CRC* genes are expressed in staminal tissues, while in other core or basal eudicots *CRC* genes are not expressed in these floral organs. Stamen expression is also found in basal angiosperms such as *C. caroliniana* and *A. trichopoda* (Fourquin *et al.*, 2005, Yamada *et al.*, 2011), although in these species *CRC* expression is detected in both filaments and anthers, while in legumes it seems restricted to the basal staminal tube. Thus, it appears more likely that *CRC* expression in stamens of basal angiosperms and legumes does not have a common origin but is the result of derived acquisition of additional regulatory elements in legumes.

In contrast, the characteristic ring-shaped expression around vascular veins was observed in the legumes in this study, and also occurs in the basal eudicot *A. formosa*, and in monocots such as rice, wheat, maize and sorghum (Yamaguchi *et al.*, 2004; Lee *et al.*, 2005; Yamada *et al.*, 2011), although in these monocot species it is not associated with carpel vasculature but found in the medial veins of lemmas, considered to be the equivalent of sepals in grasses (Ambrose *et al.*, 2000) and in the midrib of leaves. Midrib leaf expression of *CRC* genes in grasses has been generally considered to be acquired at some point at the base of monocot diversification. However, the strikingly similar expression patterns of *CRC* genes associated with the medial vein in species from such widely divergent families may suggest that elements directing expression to vasculature are ancestral but have experienced several independent modifying events that have affected their activity in the different clades, such as the gain of leaf expression in grasses and their loss in some eudicot species.

In spite of an atypical expression pattern, CRC *genes in legumes have similar roles to those in other species*

The phenotypes observed in PsCRC-VIGS plants affecting margin tissue development are related to those described for other eudicot species in which functional studies have been performed. The phenotypes in style and stigma development of the PsCRC-VIGS pea lines are of moderate severity, mainly affecting the size and morphology of these tissues, but not preventing their development, as also observed in other species in which mutation or downregulation of *CRC* has been reported (Alvarez and Smyth, 1999; Yamaguchi *et al.*, 2004; Lee *et al.*,

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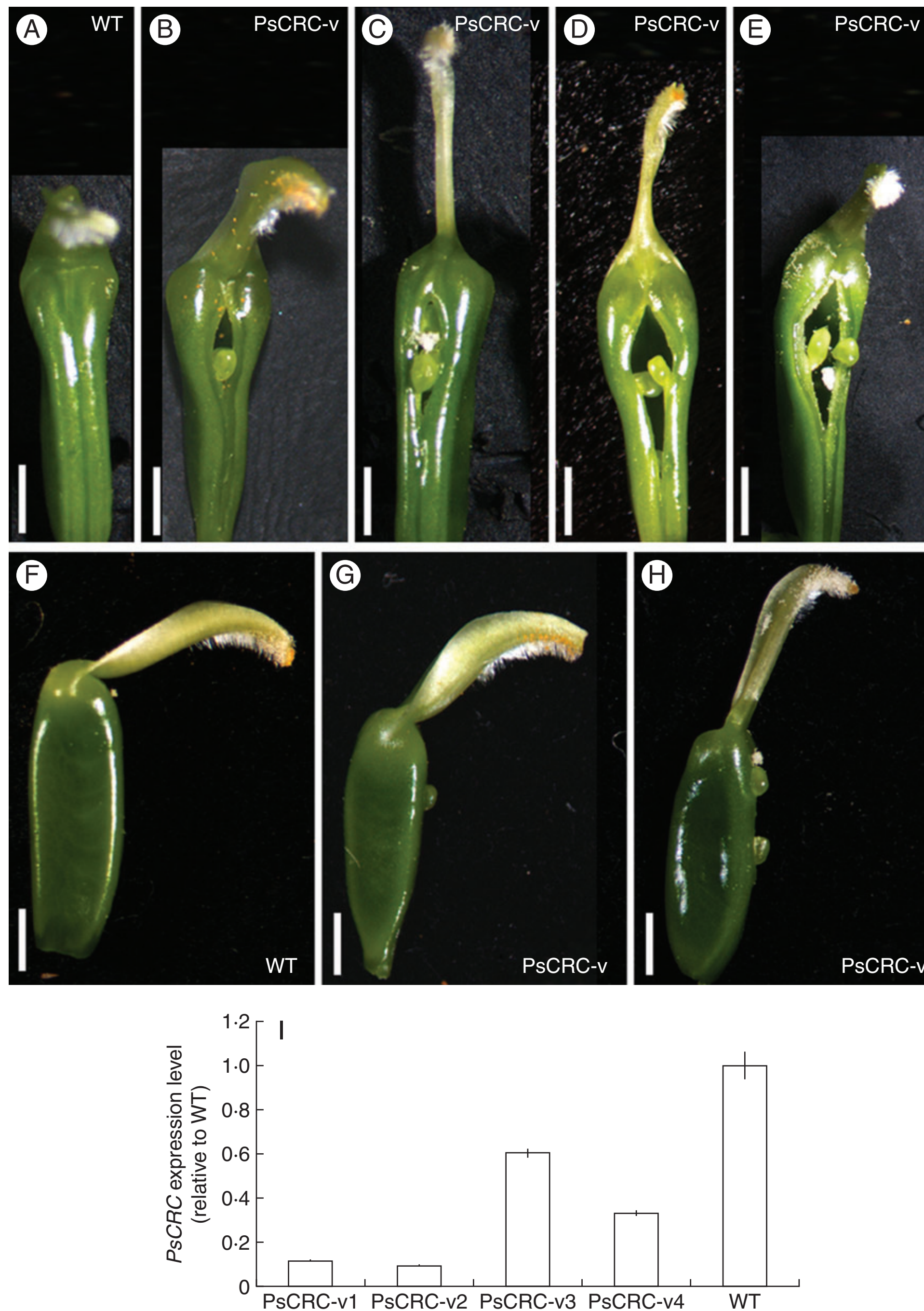

FIG. 3. Phenotypes of *P. sativum* plants inoculated with pCAPE2-PsCRC. (A–E) Ventral view of *P. sativum* pistils at anthesis. (F–H) Side view of *P. sativum* pistils at anthesis. (A, F) Wild-type pistils, composed of a flat rectangular green ovary capped by a perpendicular white elongated style, which ends with a hairy stigma. (B, G) PsCRC-VIGS pistils displaying a mild phenotype. Note unfused margins at the upper part of the ovary and the presence of an external ovule. The angle between the style and the ovary is slightly wider than in the wild-type. (C–E, H) PsCRC-VIGS pistils displaying strong phenotypes. In addition, style and stigma tissues are considerably reduced and develop almost parallel to the longitudinal axis of the ovary. Note the strongly unfused ovary and the presence of several external ovules. (I) Expression level by real-time PCR analysis of *PsCRC* in PsCRC-VIGS anthesis flowers. Values have been normalized to wild-type. Scale bar = 2 mm.

2005; Orashakova *et al*., 2009). This supports a non-essential role of *CRC* for the specification of the apical domain of the legume carpel, although it could also be possible that it relies on the residual activity of *PsCRC* in the VIGS-treated plants.

In addition to style and stigma phenotypes, PsCRC-VIGS pistils consistently showed clear defects in carpel margin fusion, again a common aspect shared by *crc* mutants in other eudicot species (Alvarez and Smyth, 1999; Lee *et al*., 2005, Orashakova *et al*., 2009). However, unlike *CRC* orthologues from these other species, *PsCRC* transcripts are not detected at or adjacent to carpel margins, suggesting that CRC may have a non-cell-autonomous activity. In fact, such non-cell-autonomous

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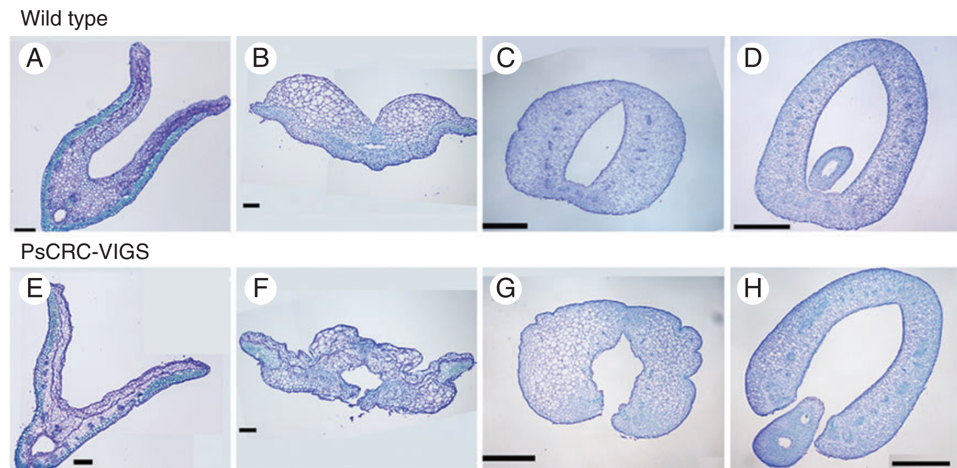

FIG. 4. Transverse sections of *P. sativum* pistils at anthesis from plants inoculated with pCAPE2-PsCRC. (A–D) Wild-type pistils. (E–H) PsCRC-VIGS pistils. (A, E) style, (B, F) transition style/ovary, (C, G) upper part of ovary, (D, H) middle part of ovary. Scale bar (A, B, E, F) = 100 µm; (C, D, G, H) = 500 µm.

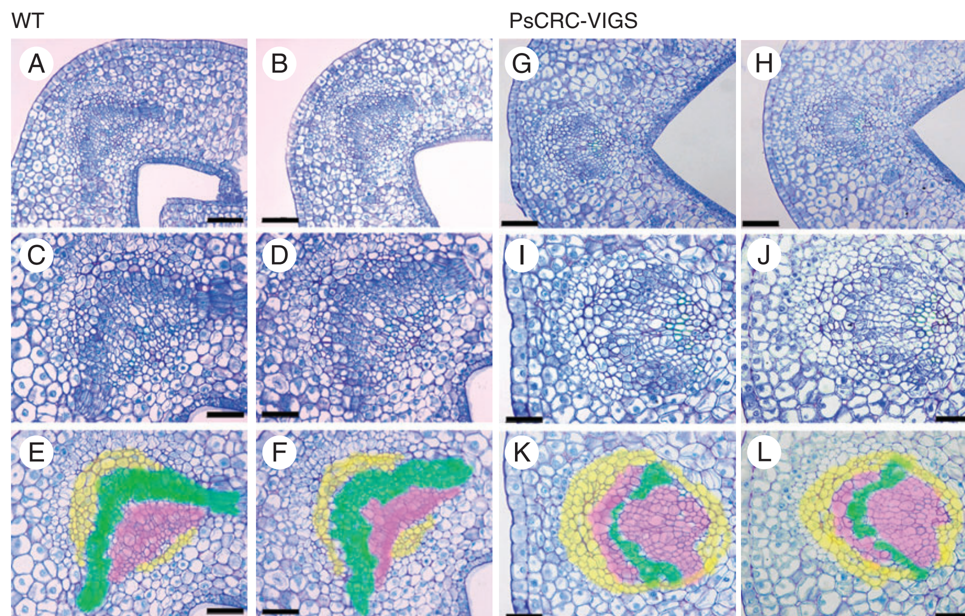

FIG. 5. Morphology of the medial vein in the ovary of PsCRC-VIGS pistils. (A–F) Transverse sections of wild-type pistils at the medial vascular bundle. (A, C, E) show the same pistil. In (C), the same section as in (A) is shown at higher magnification. In (E), the same section is shown, but vascular components have been colour-highlighted for clarity; xylem/protoxylem has been shaded in pink, phloem in green and fibres in yellow. (B, D, F) Equivalent sections to (A, C, E) for a different wild-type pistil. (G–L) Transverse sections of PsCRC-VIGS pistils at the medial vascular bundle. (G, I, K) show the same PsCRC-VIGS pistil. In (I), the same section as in (G) is shown at higher magnification. In (K), the same section is shown, but vascular components have been colour-highlighted as described above. (H, J, L) Equivalent sections to (G, I, K) for a different PsCRC-VIGS pistil. Scale bars (A, B, F, G) = 100 µm; (C–F, I–L) = 50 µm.

signalling mechanisms have already been proposed for the *E. californica* CRC orthologue (Orashakova *et al*., 2009) and other YABBY family members, such as the *Antirrhinum majus GRAMINIFOLIA* gene and the arabidopsis *FILAMENTOUS FLOWER* gene, where it does not seem to involve mRNA or protein movement directly, but to be mediated by other independent factors (Golz *et al*., 2004; Goldshmidt *et al*., 2008).

PsCRC-VIGS flowers did not show defects in floral determinacy, a phenotype that has been described in mutants for *CRC*-like genes in several eudicot and monocot species (Alvarez and Smyth, 1999; Yamaguchi *et al*., 2004; Lee *et al*., 2005; Orashakova *et al*., 2009). Although this might suggest that PsCRC does not have a role in the control of pea flower determination, other alternative scenarios are possible. For instance, it could be a consequence of the incomplete inactivation of *CRC* function in the VIGS-treated lines. Alternatively, it may reflect the possible redundancy of legume *CRC* and other factors, as observed, for example, in arabidopsis (Alvarez and Smyth, 1999; Prunet *et al*., 2008). However, it is interesting to note that flower ontogeny in pea has some particularities regarding flower termination that could be relevant to this point. In contrast to floral ontogeny in most eudicot species, in which floral organ

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initiation proceeds sequentially from sepals to petals, stamens and finally carpels, in pea the carpel primordium is initiated before petals and stamens, and therefore floral termination does not appear to be restricted to growth suppression in the centre of the floral meristem, but also involves termination of some characteristic meristematic domains known as common primordia, from which petals and stamens develop (Tucker, 1989; Ferrándiz *et al*., 1999). In fact, some indeterminate mutants characterized in pea show petal and stamen proliferation but no significant effect on carpel number (Ferrándiz *et al*., 1999). It is thus possible that *CRC* retains the carpel determination function in legumes but that early carpel differentiation reduces the ability of the flower to produce extra inner whorls in the absence of factors promoting meristem determination.

Finally, in the PsCRC-VIGS plants, we observed weak phenotypic defects in the development of the medial vein in the carpel, a phenotype not previously described for *crc* mutants in core eudicots. Interestingly, *PsCRC* expression was associated with the adaxial side of the vein, while *PsCRC* downregulation caused mainly defects in the abaxial side of the bundle. Again, this could be explained by non-cell-autonomous activity of PsCRC, although it could also be the consequence of altered cell proliferation or cell identity specification in the adaxial domain, which could have an effect on overall vein organization. Vascular phenotypes have been described for *crc* mutants in Poaceae and *E. californica*, although restricted to the midrib of leaves in grasses and to carpel vasculature in Californian poppy (Yamaguchi *et al*., 2004; Orashakova *et al*., 2009). The different nature of the organs affected in each case (vegetative in grasses, floral in poppies) has led to the proposal that *CRC* orthologues went through neofunctionalization to acquire independent roles in vascular development in both groups (Preston *et al*., 2011). However, the similarity of expression patterns around vascular tissues in monocots, basal dicots and legumes, together with the related phenotypes of the different mutants in these groups, might suggest a common origin of the role of CRC in vascular development that predates the divergence of monocots and dicots, a hypothesis whose confirmation will have to await additional studies in a larger number of representative species.

Conclusions

In this work, *CRC* orthologues from two legume species were studied by expression analyses and one of them, *PsCRC*, by the characterization of phenotypes associated with reduced CRC activity. It is concluded that the orthologues of *CRC* from pea show conserved roles in carpel development in this legume species, including carpel margin fusion and style and stigma development. In addition, expression data support the idea that CRC factors from legume species also direct nectary development, a common feature of *CRC* genes in all eudicot species in which functional studies are available. Despite these conserved roles, *CRC* genes from legumes show atypical expression patterns in carpel development, which also supports the hypothesis of CRC acting non-cell-autonomously. An intriguing result of this work is the likely role of *CRC* in vascular development, which appears to be shared by species of monocots, basal eudicots and core eudicots, challenging the generally accepted idea of the neofunctionalization of *CRC* orthologues to direct leaf midrib development in monocots.

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