

RESEARCH PAPER

***Ca*JOINTLESS is a MADS-box gene involved in suppression of vegetative growth in all shoot meristems in pepper**

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Abstract

In aiming to decipher the genetic control of shoot architecture in pepper (*Capsicum* spp.), the allelic late-flowering mutants *E-252* and *E-2537* were identified. These mutants exhibit multiple pleiotropic effects on the organization of the sympodial shoot. Genetic mapping and sequence analysis indicated that the mutants are disrupted at *Ca*JOINTLESS, the orthologue of the MADS-box genes *JOINTLESS* and *SVP* in tomato and *Arabidopsis*, respectively. Late flowering of the primary and sympodial shoots of *Cajointless* indicates that the gene functions as a suppressor of vegetative growth in all shoot meristems. While *Ca*JOINTLESS and *JOINTLESS* have partially conserved functions, the effect on flowering time and on sympodial development in pepper, as well as the epistasis over *FASCICULATE*, the homologue of the major determinant of sympodial development *SELF-PRUNING*, is stronger than in tomato. Furthermore, the solitary terminal flower of pepper is converted into a structure composed of flowers and leaves in the mutant lines. This conversion supports the hypothesis that the solitary flowers of pepper have a cryptic inflorescence identity that is suppressed by *Ca*JOINTLESS. Formation of solitary flowers in wild-type pepper is suggested to result from precocious maturation of the inflorescence meristem.

Key words: Flowering time, jointless, pepper, shoot architecture, sympodial growth, tomato.

Introduction

Higher plants exhibit great variation in their shoot architecture, manifested by degree of lateral branching, internode elongation, leaf complexity, determinacy, and inflorescence structure (Alonso-Blanco *et al.*, 2009). Similarly, large variation exists in flowering time and in the mechanisms that control the transition to flowering. Isolation of mutants affected in flowering time or plant architecture has revealed that these two aspects of development are frequently interconnected. The genetic control of plant architecture and flowering time has been studied mostly in a limited number of model species, such as *Arabidopsis*, rice, maize, tomato, and petunia (Izawa, 2007; Wang and Li, 2008; Michaels, 2009; Yant *et al.*, 2009; Castel *et al.*, 2010). However, while model species are imperative to defining the developmental pathways and genes regulating plant architecture, the extent to which these genes and pathways are conserved in non-model crop species has yet to be determined.

In contrast to *Arabidopsis* that exhibits a monopodial shoot system in which the shoot apical meristem (SAM) is indeterminate – first producing leaves and later flowers – Solanaceae plants such as pepper, tomato, and petunia exhibit a sympodial growth architecture. Sympodial growth is characterized by the determinacy of the SAM, which converts from vegetative to reproductive meristem comprised of a terminal flower. In pepper, the meristem is arrested at that stage and vegetative sympodial branches are released from basal leaves. In tomato, basal nodes below the flower branch to form another flower, in a reiterative process, leading to the formation of a compound inflorescence made up of several flowers organized in a zigzag pattern (Lifschitz and Eshed, 2006). In both shoots, however, further growth continues from the uppermost axillary meristem located at the node below the inflorescence or flower of the primary stem; this meristem is referred to

as sympodial meristem (SYM). Similar to the primary SAM, the SYM terminates after producing a short shoot segment, termed the sympodial unit, consisting of three leaves and an inflorescence in tomato and two leaves and a solitary flower in pepper. Further growth of the sympodial shoot continues from a lateral meristem at the axil of the uppermost leaf of the SYM. These sympodial units are reiterated to form the sympodial shoot structure.

Detailed studies on the regulation of flowering in *Arabidopsis* have revealed several pathways that control the transition from vegetative to reproductive development (Michaels, 2009; Yant *et al.*, 2009). These include pathways that are environmentally dependent on temperature and day length or on gibberellin, and an autonomous pathway that mediates flowering by monitoring the developmental stage of the plant. To date, more than 100 genes have been identified as regulating this process in *Arabidopsis* (Srikanth and Schmid, 2011). In cultivated tomato and pepper, which do not respond to photoperiod or vernalization signals (Samach and Lotan, 2007), the autonomous pathway is thought to play a major role in the transition to flowering. However, the molecular mechanisms that underlie the day-neutral flowering response of these plants are just beginning to be revealed (Mizoguchi *et al.*, 2007).

Florigen is a mobile flowering-initiation signal produced in the leaves that induces flowering at the SAM. The identity of florigen was first established as the *FLOWERING LOCUS T* (*FT*) homologue *SINGLE FLOWER TRUSS* (*SFT*) in tomato, (Lifschitz *et al.*, 2006). Subsequently, the role of *FT* homologues as florigens in other plant species, such as rice, *Arabidopsis*, and maize, was established as well (Tamaki *et al.*, 2007; Meng *et al.*, 2011). While *FT* homologues appear to have a universal function as florigens, their precise action is species specific (Wigge, 2011). Furthermore, the *FT/SFT*-paralogous genes *TERMINAL FLOWER1* (*TFL1*) and *SELF PRUNING* (*SP*) in *Arabidopsis* and tomato, respectively, have antagonistic functions similar to *FT* homologues in determining plant shoot determinacy (Conti and Bradley, 2007; Shalit *et al.*, 2009). *TFL1/SP* homologues were subsequently found to have a species-specific adapted role in controlling plant architecture in diverse plants, such as soybean, poplar, and pepper (Elitzur *et al.*, 2009; Liu *et al.*, 2010; Mohamed *et al.*, 2010; Tian *et al.*, 2010). An emerging conclusion from these and other studies is that, although genes controlling architecture and flowering are common to different plant species, their function may be modified in a species-specific manner.

FT is negatively regulated by a complex of MADS-box genes that includes *FLOWERING LOCUS C* (*FLC*) and *SHORT VEGETATIVE PHASE* (*SVP*) (Li *et al.*, 2008). *SVP* is a negative regulator of flowering time (Hartmann *et al.*, 2000) and functions in the floral meristem by activating the floral class B and C homeotic genes that specify floral organ identity (Liu *et al.*, 2009). The tomato orthologue of *SVP* is *JOINTLESS* (Mao *et al.*, 2000). The *jointless* mutant is characterized by reversion of the inflorescence after production of a few flowers into a vegetative shoot (Szymkowiak and Irish, 2006). Analysis of the interactions of *jointless* with other mutants controlling meristem identity in tomato revealed that the inflorescence and SYMs share common regulatory mechanisms. In addition to its function in inflorescence development, *JOINTLESS* interacts with the MADS-box gene *MACROCALYX* to regulate development of the abscission zone in the flower pedicel (Nakano *et al.*, 2012).

Pepper (*Capsicum* spp.) is an important solanaceous crop, but despite the importance of shoot architecture to crop production, the genetic basis of its variation in growth habit has been poorly explored. Therefore, developmental mechanisms and genes controlling growth habit and transition to flowering in that species remain to be deciphered and the extent to which these genes and pathways are conserved with related model species determined. Because only limited natural variation for these traits exists in pepper, an ethyl methanesulphonate (EMS)-mutagenized population was generated on the background of the blocky-fruited cultivar 'Maor' and screened for alterations in plant architecture (Paran *et al.*, 2007). In the present study, the isolation of a late-flowering mutation that is disrupted at *CaJOINTLESS*, which functions as a floral promoter in primary and sympodial shoots, is reported. Moreover, *CaJOINTLESS* has a role in suppressing inflorescence development and maintaining the single-flower organization of the pepper truss.

Materials and methods

Plant material

The mutants *E-252*, *E-2537*, *Cablind* (*Cabl*) and *Ca-anantha* (*Ca-an*) were isolated from an EMS mutagenesis population using *Capsicum annum* cv. 'Maor' as a wild-type parent (Paran *et al.*, 2007). *Cabl* and *Ca-an* have been described by Jeifetz *et al.* (2011) and by Lippman *et al.* (2008), respectively. *fasciculate* (*fa*) is a spontaneous mutation described by Elitzur *et al.* (2009). To map the *E-252* mutation, a segregating BC₁ population was constructed from a cross of *E-252* and *Capsicum chinense* PI 159234 using *E-252* as the recurrent parent.

Double mutants of *E-252:Cabl* and *E-252:fa* were derived from F₂ populations obtained by crossing the corresponding mutants and screening by cleaved amplified polymorphic sequence markers for double mutations. To generate the double mutant *E-252:Ca-an*, crosses were performed between *E-252* and a heterozygous *Ca-an/+* because fertile flowers are not produced in *Ca-an*, followed by screening an F₂ population for double mutants.

Tomato (*Solanum lycopersicum*) *jointless* (*j*) seeds (LA3023) were obtained from the TGRC stock centre at UC Davis, California. Seeds of cv. M82 *SP* (wild type), M82 *sp*, and *j:sp* double mutants on the background of M82 were obtained from Y. Eshed, The Weizmann Institute, Israel.

Isolation of *CaJOINTLESS* (*CaJ*)

To map *E-252*, selected individuals of mutant and wild-type phenotypes from the BC₁ segregating population were used for screening with RFLP markers scattered throughout the genome, according to Ben Chaim *et al.* (2001). To determine linkage between *E-252* and *CaJ*, tomato *JOINTLESS* (accession no. AF275345) was amplified with the primers Tom-JF and Tom-JR using tomato genomic DNA as the template (Table 1), and the amplified product was used as an RFLP probe in Southern blot analysis. The same primers were used to amplify a corresponding partial fragment from pepper genomic DNA. A 1502-bp fragment was cloned into pDrive PCR Cloning Vector (Qiagen) and used to screen a bacterial artificial chromosome (BAC) library of *Capsicum frutescens* BG 2816 obtained from the Arizona Genomics Institute (<http://www.genome.arizona.edu/orders>). A positive BAC clone (0215J14) was sequenced at the genome centre at Washington University, St. Louis, Missouri. Subsequently, the genomic region containing *CaJ* identified by BLAST was sequenced in 'Maor' and *E-252*. RNA isolated from leaves was used to create cDNA of the mutant and wild type, which was used as a template for sequencing the open reading frames (ORFs) using primers matching the 5' and 3' untranslated regions, CaJ-5UTR and CaJ-3UTR, respectively (Table 1). The ORF of *CaJ* from 'Maor' was deposited in GenBank with accession number JQ698661. For screening the *CaJ* mutation in subsequent experiments, a cleaved amplified polymorphic sequence marker was developed by using PCR primers Tom-JF and CaJ-465R (Table 1) followed by restriction digestion with *Hph*I.

Table 1. Primers used in this study

Gene	Primer	Primer sequence (5'-3')
JOINTLESS	Tom-JF	GATCTCTTGAAGACTGGATTGAGCC
	Tom-JR	TFACTTTTTTTTTCTCCTCTTCTAATAAC
	J-qRTF	TCAATTGATCCTCCTCCTCAA
	J-qRTR	AGCCACACCTTGCTTTTGAT
CaJOINTLESS	CaJ-5UTR	GAAAGAAAGACTCGTAATGGC TAGAGAGAAAATT
	CaJ-3UTR	TTAAGCCAAGTTGAATAAAAAGACTTC
	CaJ-465R	GTTGGAGCTGATTAATCTCTCATTAA
	CaJ-qRTF	TTAAGGCAAATGAGGGGTGA
FASCICULATE	FA-qRTF	CCAGCAGAAGAAAAGGCCAAAC
	FA-qRTR	AGCTACAGGAGAGCCAAGTTC
SELF-PRUNING	SP-qRTF	TATCGAGTGCACCAAGTGTCC
	SP-qRTR	CCTTCTAGCGGCAGTTTCC
CaUBIQUITIN	UBQ-qRTF	GCACAAGCACAAGAAGGTAAAG
	UBQ-qRTR	GCACCACACTCAGCATTAGGA
UBIQUITIN3	UBC3-qRTF	GGTCTGTGTGCCATTGTCT
	UBC3-qRTR	GTCTCGTATTGGCCCTGTC

Analysis of gene expression

Total RNA was extracted from shoot apices using GeneElute Mammalian Total RNA Extraction Miniprep kit (Sigma) according to the manufacturer's instructions, followed by DNase I treatment (Sigma). Total RNA (400 ng) was used for first-strand cDNA synthesis by reverse-transcription PCR (RT-PCR) using a PrimeScript RT Reagent kit (Takara). For quantitative real-time PCR (qRT-PCR), three biological and two technical repeats were used for each sample. For the qRT-PCR experiments, plants were grown in a glasshouse under natural daylight during the winter season in Israel. PCR amplification was performed using the primers CaJ-qRTF and CaJ-qRTR for *CaJ* and FA-qRTF and FA-qRTR for *FA* (Table 1). Expression analysis of tomato genes was carried out using the primers J-qRTF and J-qRTR for *JOINTLESS* and SP-qRTF and SP-qRTR for *SP*. Amplified products were detected using SYBR Premix Ex Taq II (Takara) in a Rotor-Gene 6000 thermal cycler (Corbett). Results were analysed using Rotor-Gene 6000 Series software 1.7 (Corbett). The relative expression levels of the pepper and tomato genes were normalized against, respectively, pepper *CaUBIQUITIN* (DQ975458.1) using the primers UBQ-qRTF and UBQ-qRTR, and tomato *UBIQUITIN3* (FJ429328.1) using the primers UBC3-qRTF and UBC3-qRTR.

in situ hybridization

Digoxigenin (DIG)-labeled *in situ* analysis was performed as previously described (Szymkowiak and Irish, 2006; Neta et al., 2011). Meristems were fixed in FAA (formaldehyde/acetic acid/70% ethanol, 10:5:85, v/v), dehydrated and embedded in ParaPlast (McCormick Scientific). The tissue was then cut (10 µm) on a Leica RM2245 microtome (VectaMount, Vector Laboratories) and sections were placed on SuperFrost Plus slides (Menzel-Glaser) for 2 days on a 42 °C hot plate. An antisense DIG-labelled RNA probe was synthesized from the 3' end of *CaJ* cDNA, which excludes the conserved domains, using the MEGAscript kit (Ambion) and DIG RNA labelling mix (Roche Applied Science).

Scanning electron microscopy (SEM)

Samples for SEM were fixed directly in 70% ethanol and critically point dried as described by Alvarez et al. (1992). SEM was performed using a Hitachi S-3500N instrument.

Phylogenetic analysis

Multiple sequence alignments were performed with a web-based version of Clustal W (<http://www.ebi.ac.uk/Tools/clustalw/>) using the

default settings. The phylogenetic tree was calculated using the neighbour-joining method and bootstrap analysis with 1000 replicates via MEGA4 software (<http://www.megasoftware.net/mega.html>). The tree was calculated from alignments of the MADS-box and K-box homeodomains, consisting of the first ~170 amino acids of the proteins.

Results

Pepper shoot architecture and its modifications

Pepper has a sympodial shoot architecture, producing about 10 leaves on the primary stem before termination with a single flower (Fig. 1A). After termination of the primary shoot, further growth occurs from the axils of the two uppermost leaves (Fig. 1B). A SYM differentiates in each axil, which develops into a sympodial unit that consists of two leaves and a single flower (Fig. 1B, 1C). The two leaves grow away from the flower and are positioned above it. The initiation of SYMs in the two leaf axils results in a bifurcated shoot (Fig. 1C). Additional cycles of sympodial development continue from meristems formed at the axils of the two leaves. These cycles of development repeat themselves and can theoretically continue indefinitely.

Phenotypic screening of an EMS-mutagenized population of the blocky cultivar 'Maor' for mutants with altered growth habit identified *E-252*, which exhibits multiple pleiotropic changes in its growth habit that are inherited as a single recessive trait. The *E-252* mutant is indistinguishable from the wild type during the vegetative phase and there is no architectural difference between the mutant and wild-type plants before flowering. However, whereas wild-type plants shift from vegetative to reproductive phase after an average of 10 leaves on the primary stem, the mutant plants flower later, after an average 17 leaves. The sympodial units of *E-252* often have an extra leaf or two (Fig. 1D, 1E) and the bifurcated shoot architecture is maintained by the two uppermost leaf axils. Later, during sympodial growth, the entire sympodial unit is carried away from the flower and located above it (Fig. 1G). During floral development, two or more flowers may form on the same pedicel with the appearance of vegetative growth along them, resembling the formation of a vegetative inflorescence (Fig. 1F). At flower development, one of the sepals is often converted into a leaf-like organ (not shown).

E-252 is disrupted at *CaJ*

To isolate the gene governing the *E-252* mutation, a BC₁ segregating population was screened with RFLP markers scattered throughout the pepper genome. One tomato-originated marker, TG194, mapped to chromosome 12 of pepper (Livingstone et al., 1999), was found tightly linked to the mutation (not shown). Searching for genes associated with tomato architecture mapped near this marker enabled detection of the MADS-box transcription factor gene *JOINTLESS* (*J*) (Mao et al., 2000; Szymkowiak and Irish, 2006) as a potential candidate for controlling the *E-252* mutation. RFLP mapping of *J* in the BC₁ population of 15 mutant and 15 wild-type individuals indicated complete co-segregation with the mutant phenotype. The sequence of the pepper orthologue of *JOINTLESS* (*CaJ*) was isolated using cDNA, BAC, and genomic DNA sources and encodes a 234-amino-acid protein (Fig. 2A). Comparison of cDNA and genomic sequences revealed

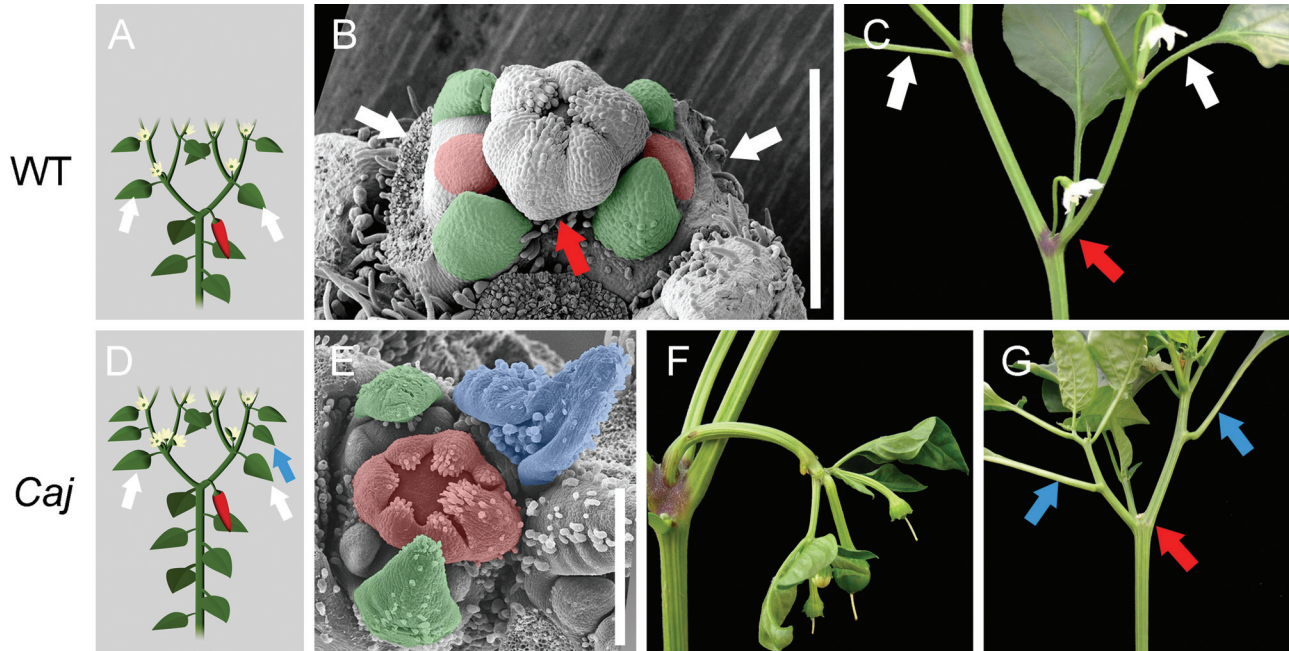


Fig. 1. Characterization of wild-type pepper and *E-252* mutant. (A) Schematic diagram of wild-type parent ‘Maor’. The two leaves that belong to the first sympodial unit are indicated by white arrows. (B) Scanning electron micrograph of wild-type shoot apical meristem, showing a flower (red arrow), locations of flanking leaves (excised, white arrows), and two sympodial meristems, each consisting of two leaf primordia (green) and a flower meristem (red). (C) Wild-type sympodial unit consisting of a flower (red arrow) and two leaves (white arrows). (D) Schematic diagram of *E-252* mutant. The extra sympodial leaf is indicated by a blue arrow. (E) Scanning electron micrograph of *E-252* sympodial meristem consisting of a flower (red), two leaf primordia (green), and an additional primordial leaf (blue) with no developed meristems in its axil. (F) *E-252* flower pedicel carrying multiple flowers and leaves. (G) *E-252* sympodial unit consisting of a flower (excised, red arrow) and two extra leaves (blue arrows). Bars, 400 μ m.

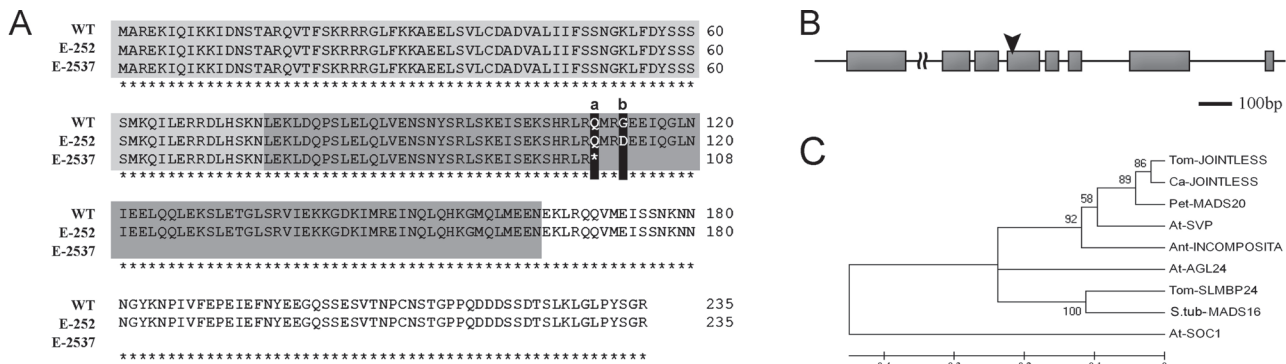


Fig. 2. Molecular characterization of *CaJ*. (A) Amino acid sequence of the *CaJ* protein. The MADS-box domain is shaded in light grey and the K-box motif is shaded in dark grey. The mutations in *E-2537* and *E-252* are indicated by a and b, respectively. (B) Schematic description of *CaJ*. Introns and exons are indicated by solid lines and grey boxes, respectively. The region containing the EMS mutations in exon 4 is indicated by an arrowhead (note that the size of the first intron is larger than 2 kb and is not to scale). (C) Phylogenetic tree of the conserved domains of *JOINTLESS*-related proteins from the subclade family *StMADS11* and *SOC1* from *Arabidopsis*. Accession numbers: *AGL24*, NP_194185.1; *INCOMPOSITA*, CAG27846.1; *JOINTLESS*, AAG09811.1; *MADS16*, AAV65504.1; *MADS20*, ACY82403.1; *SLMBP24*, TC161634; *SOC1*, NP_182090.1; *SVP*, NP_001154528.1.

that the gene consists of eight exons (Fig. 2B). Sequencing *CaJ* in *E-252* identified a missense mutation of G to A at position 338 of the ORF that results in a change of glycine to aspartic acid at position 114 of the protein (Fig. 2A). To prove that *CaJ* controls the *E-252* mutation, the EMS population was screened for additional mutants showing a phenotype similar to *E-252*. This search identified *E-2537* which was defined as allelic to

E-252 by allelism test (F_1 from a cross between the two recessive mutants had a mutant phenotype). Sequencing of *E-2537* indicated a single nucleotide change of C to T at position 328 of the ORF, resulting in the formation of a stop codon at position 110 of the protein (Fig. 2A). Both mutations are located in exon 4 in the K-box motif that is involved in a protein–protein interaction of type II MADS-box genes (Egea-Cortines *et al.*, 1999).

CaJ contains two conserved domains at its N-terminal end (Fig. 2A) and belongs to the StMADS11 subclade (García-Maroto *et al.*, 2000; Becker and Theissen, 2003). Phylogenetic analysis showed that CaJ is closely related to tomato J, and together they are clustered in the same clade with *Arabidopsis* SVP, *Petunia* MADS20 and *Antirrhinum* INCOMPOSITA (Fig. 2C).

CaJ is expressed in all shoot meristems

The expression level of *CaJ* was analysed in different plant tissues. The strongest expression level was observed in sympodial

apices, followed by flower buds and young leaves, whereas no expression was observed in mature flower petals (Fig. 3A). The spatial and temporal expression of *CaJ* was further explored using qRT-PCR and *in situ* hybridization. Sampling mRNA from shoot apices at different stages of development in 'Maor' and *E-252* indicated that *CaJ* is already expressed before the transition to flowering (2-expanded leaf stage; Fig. 3B). Transcript level was highest just before transition to flowering (4-leaf stage) and declined later on (8- to 12-leaf stages). The expression pattern in *E-252* resembled that of 'Maor' although at a lower level, indicating possible auto-regulation of *CaJ*. *In situ*

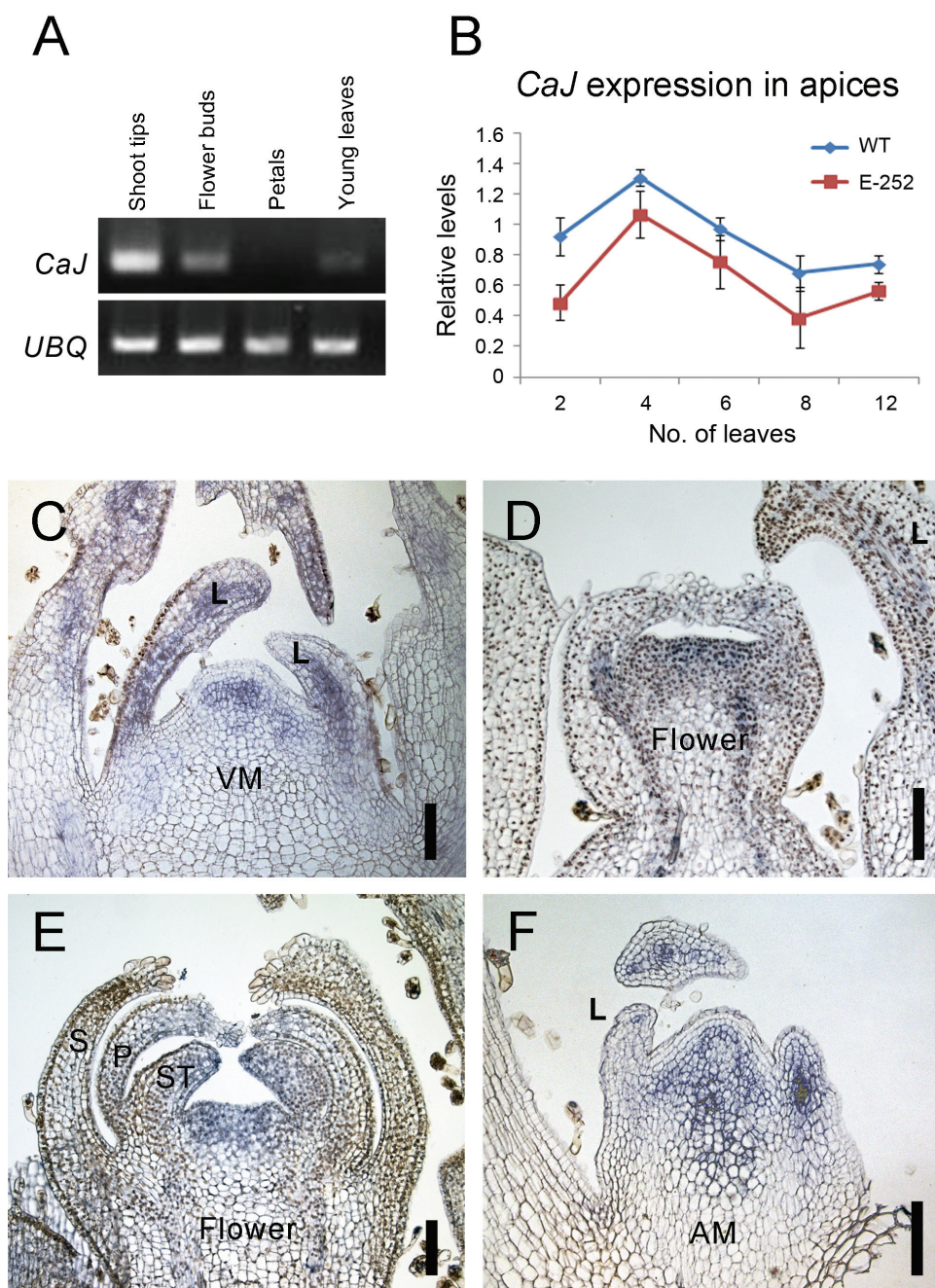


Fig. 3. Expression pattern of *CaJ*. (A) Reverse-transcription PCR of *CaJ* transcripts in different tissues of 'Maor'. (B) Expression of *CaJ* transcripts in wild-type and mutant apices at different leaf stages on the primary stem. (C–F) *In situ* hybridization of *CaJ* transcripts in vegetative apex at 2-leaf stage (C), young flower at 6-leaf stage, (D), flower at 8-leaf stage (E), and axillary meristem (F). Bars, 100 μ m. AM, axillary meristem; L, leaf; P, petal; S, sepal; ST, stamen; VM, vegetative meristem.

hybridization analysis revealed *CaJ* transcripts in the central zone of the SAM at the vegetative stage, as well as in the leaf primordia (Fig. 3C). *CaJ* transcripts were also detected in floral meristems at different stages. In the early flowering meristem, expression was observed in the central meristematic zone but not in the sepal primordia (Fig. 3D). At a more advanced stage of flower development, expression was observed in the central zone and in the petal and stamen primordia but not in the sepal primordia (Fig. 3E). In addition, expression was detected in the axillary meristems (Fig. 3F).

CaJ and *Ca-AN* independently control floral meristem identity

Because *Caj* mutants are altered in their transition to flowering, floral meristem identity, and sympodial development, the existence of a regulatory hierarchy between *CaJ* and other genes affecting these processes in pepper was explored. Double mutants of *Caj* with other available mutants affected

in transition to flowering, floral meristem identity, or sympodial development were created. These included *Ca-anantha* (*Ca-an*), *fasciculate* (*fa*), and *Cablind* (*Cabl*), controlled by the orthologues of tomato *ANANTHA*, *SELF PRUNING* and *BLIND*, respectively (Lippman *et al.*, 2008; Elitzur *et al.*, 2009; Jeifetz *et al.*, 2011).

Ca-AN is required for normal differentiation of flower organs. The *Ca-an* mutant produces an indeterminate shoot-like structure instead of a single flower, resulting in a cauliflower-like inflorescence (Fig. 4A). No change in flowering time is observed in the mutant compared to ‘Maor’ (Lippman *et al.*, 2008). The double mutant *Caj:Ca-an* had a similar late-flowering phenotype and an extra leaf in the sympodial unit as in *Caj*. The floral meristem produced an indeterminate shoot consisting of leaves and anantha-like structures (Fig. 4D), combining the floral characteristics of both mutants. Therefore, these phenotypes suggest that *Caj* and *Ca-an* have additive effects and that their corresponding genes operate in independent pathways to promote floral meristem identity.

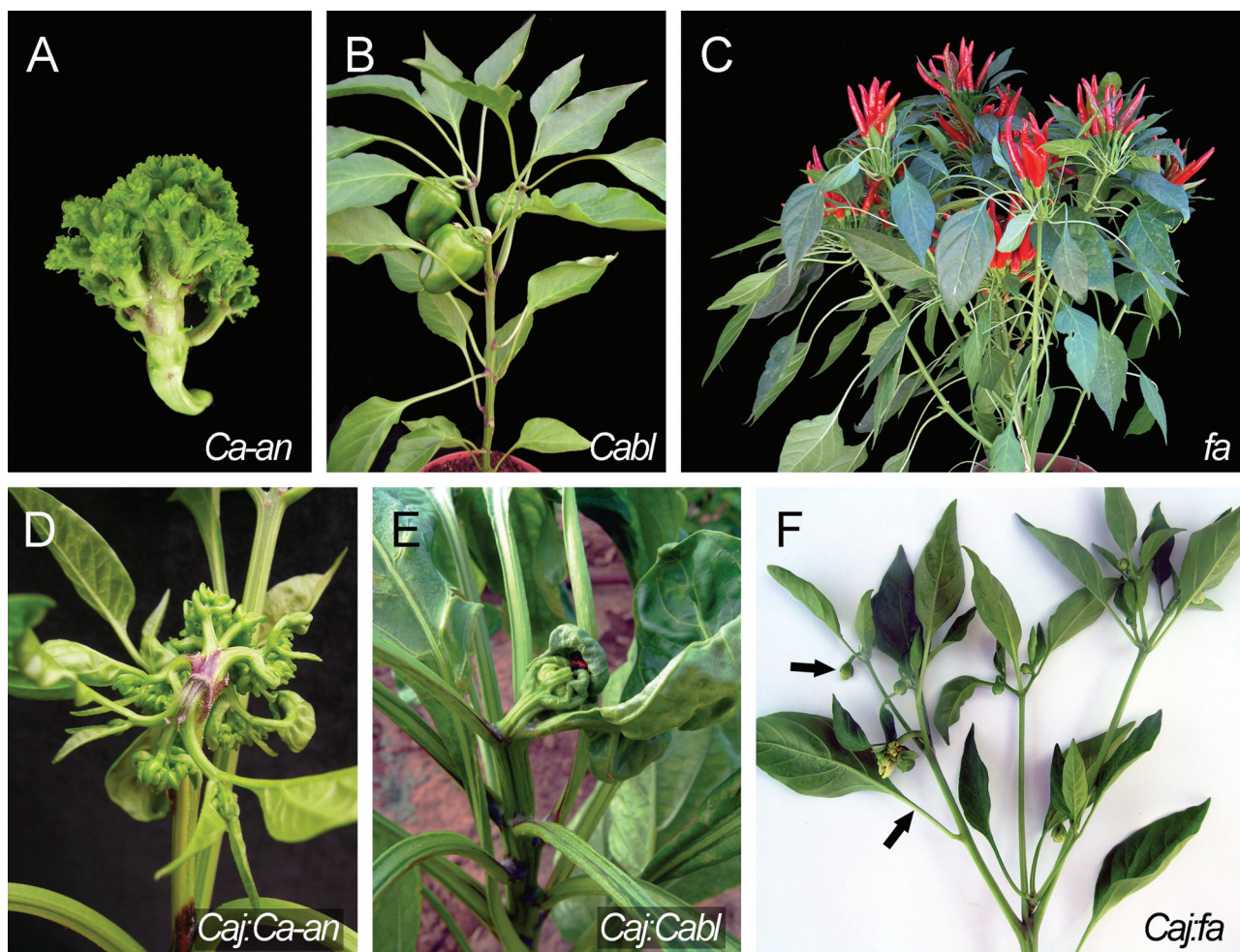


Fig. 4. Pepper mutants affected in growth habit and their interaction with *Caj*. (A) *Ca-an* indeterminate shoot-like inflorescence. (B) *Cabl* mutant exhibits no axillary growth and early termination of sympodial growth. (C) *fa* mutant exhibits fruit clusters and determinate growth. (D) *Caj:Ca-an* exhibits vegetative indeterminate inflorescence structure. (E) *Caj:Cabl* exhibits late flowering, lack of axillary meristems, and early termination of the sympodial shoot by a single flower. (F) *Caj:fa* sympodium resembles *Caj* single mutant characteristics. Arrows indicate extra sympodial leaf and single flower.

CaJ and CaBL interact with each other in controlling flowering time and sympodial development

CaBL is required for lateral meristem differentiation and normal sympodial development. *Cabl* is characterized by a lack of axillary meristems in the primary shoot and precocious termination of the sympodial shoot after development of a few sympodial units. In addition, transition to flowering is delayed by 5.5 leaves compared to ‘Maor’, one of the sepals is converted into a leaf-like organ, and the sympodial unit consists of an extra leaf compared to that of ‘Maor’ (Fig. 4B; Jeifetz et al., 2011). The double mutant *Caj:Cabl* lacked axillary meristems and flowered after 30 leaves on the primary stem, producing a single terminal flower with a leaf-like sepal that completely terminated shoot growth as all axillary shoots were blocked (Fig. 4E). The synergistic late-flowering phenotype of the double mutant indicated that the two genes interact with each other in controlling the transition to flowering. Furthermore, the complete elimination of lateral shoot growth in *Caj:Cabl* indicated that the two genes redundantly regulate lateral shoot outgrowth.

CaJ is epistatic to FA in determining shoot architecture

FA is a flowering repressor in the primary and sympodial shoots and its action is antagonistic to that of *CaJ*. Pepper *fa* is characterized by early flowering time and a ‘determinate’ growth habit, resulting in the formation of floral clusters separated by short internodes and leaves (Fig. 4C). However, in *fa* plants, the basic structure of the sympodial unit remains unchanged (Elitzur et al., 2009). The phenotype of the *Caj:fa* double mutant was similar to that of *Caj*, i.e. it flowered after 17 leaves, no floral clusters were observed, the sympodial unit had an additional leaf, and occasionally, one of the sepals developed into a leaf-like organ (Fig. 4F). These phenotypes suggest that *Caj* is epistatic to *fa* with respect to development of apical, sympodial, and floral meristems.

Because of the antagonistic interaction of *CaJ* and *FA*, their interaction in controlling pepper growth habit was further examined by measuring their expression level by qRT-PCR in four different genotypes: wild type (‘Maor’) and the *Caj*, *fa*, and *Caj:fa* mutants. Expression levels were determined in the meristems of the second sympodial unit, after floral transition. Expression of *CaJ* in the *Caj* mutant was lower than in the wild type, indicating auto-regulation of *CaJ*, which is common for *SVP*-related genes (de Folter et al., 2005). Expression of *CaJ* was upregulated in the *fa* mutant compared to the wild type (Fig. 5A). In contrast, expression of *CaJ* as well as *FA* was downregulated in the *Caj:fa* mutant compared to *fa* and to the wild type (Fig. 5A, 5B). These results are in accordance with the epistatic action of *CaJ* over *FA* in the double mutant and indicate that *FA* inhibits *CaJ*.

To further study the relationship between *CaJ* and *FA* in pepper in comparison to the homologous genes in tomato, a tomato *j:sp* double mutant was obtained and the expression of the corresponding genes in the individual and double mutants was measured. The sympodial unit of wild-type tomato consists of three leaves and an inflorescence composed of multiple flowers (Fig. 5E). This structure is maintained in the sympodial unit of *j*, but the inflorescence produces a few flowers and then converts to vegetative growth (Fig. 5E). In *sp* mutants, the number

of leaves within the sympodial unit is progressively reduced until growth is terminated by two sequential inflorescences (Pnueli et al., 1998). The phenotype of *j:sp* resembled that of *sp*, i.e. the number of leaves between the inflorescences was progressively reduced and no vegetative growth was observed in the inflorescence (Fig. 5F), indicating that unlike in pepper, *sp* is epistatic to *j* with respect to sympodial growth and inflorescence development.

Expression of *J* in SYMs of tomato was upregulated in *sp* and downregulated in *j:sp* (Fig. 5C), in a pattern similar to that of *CaJ* in pepper-homologous genotypes. In contrast, the expression pattern of *SP* and its pepper homologue *FA* differed between tomato and pepper. *SP* was upregulated in *j:sp* (Fig. 5D), whereas *FA* was downregulated in the homologous *Caj:fa* pepper double mutant. These findings show that *CaJ* and its homologue from tomato have the same expression patterns in both the wild-type and homologous mutants. In contrast, the expression pattern of *SP* and *FA* is not conserved in the two species, which may contribute to the different epistatic relationship between the two genes in pepper and tomato.

Discussion

Role of CaJ in controlling transition to flowering

The *Caj* mutant was initially discovered because of its late-flowering phenotype, exhibiting a delay of seven leaves to first flower on the primary stem compared to ‘Maor’. A late-flowering phenotype was also reported for a tomato *j* mutant, but the delay in flowering was weaker than in pepper (1–3-leaf delay, depending on the genetic background, Emery and Munger, 1970; Thouet et al., 2012). Therefore, *CaJ* and *J* function to promote termination of the SAM and accelerate flowering. Interestingly, *SVP*, the closest homologue of *J* in *Arabidopsis*, is a flowering repressor operating via repression of the flowering integrator *SOC1* in the SAM and *FT* in leaves (Hartmann et al., 2000; Li et al., 2008). In contrast, *Arabidopsis AGL24*, the closest homologue of *SVP*, is a flowering promoter that activates *SOC1* (Gregis et al., 2006; Liu et al., 2008). Homologues of *SVP* in other plant species, such as *INCOMPOSITA* in *Antirrhinum* (Masiero et al., 2004), *TaVRT-2* in wheat (Kane et al., 2005), and *OsMADS55* in rice, function as flowering repressors when expressed in *Arabidopsis* (Lee et al., 2012). Other homologues from the StMADS11 subclade, such as *MPP1* from *Physalis*, function as flowering promoters (He et al., 2010). Therefore, while members of the StMADS11 subclade MADS-box genes have a conserved role in regulating flowering time in diverse plant species, their role as promoters or repressors of flowering is case specific.

The pronounced late-flowering phenotypes of *Caj* and *Caj:Cabl* indicate that the function of *CaJ* in controlling flowering time in pepper is stronger than that of its tomato homologue *J*. In addition to late flowering, *Caj* and *Cabl* share other common characteristics, including addition of leaves to the sympodial units and conversion of one sepal in the flower into a leaf-like organ. These characteristics are also common to other late-flowering pepper mutants such as *E-172-1* (data not shown). The increase in vegetativeness in different parts of the shoot in these mutants indicates that all shoot meristems,

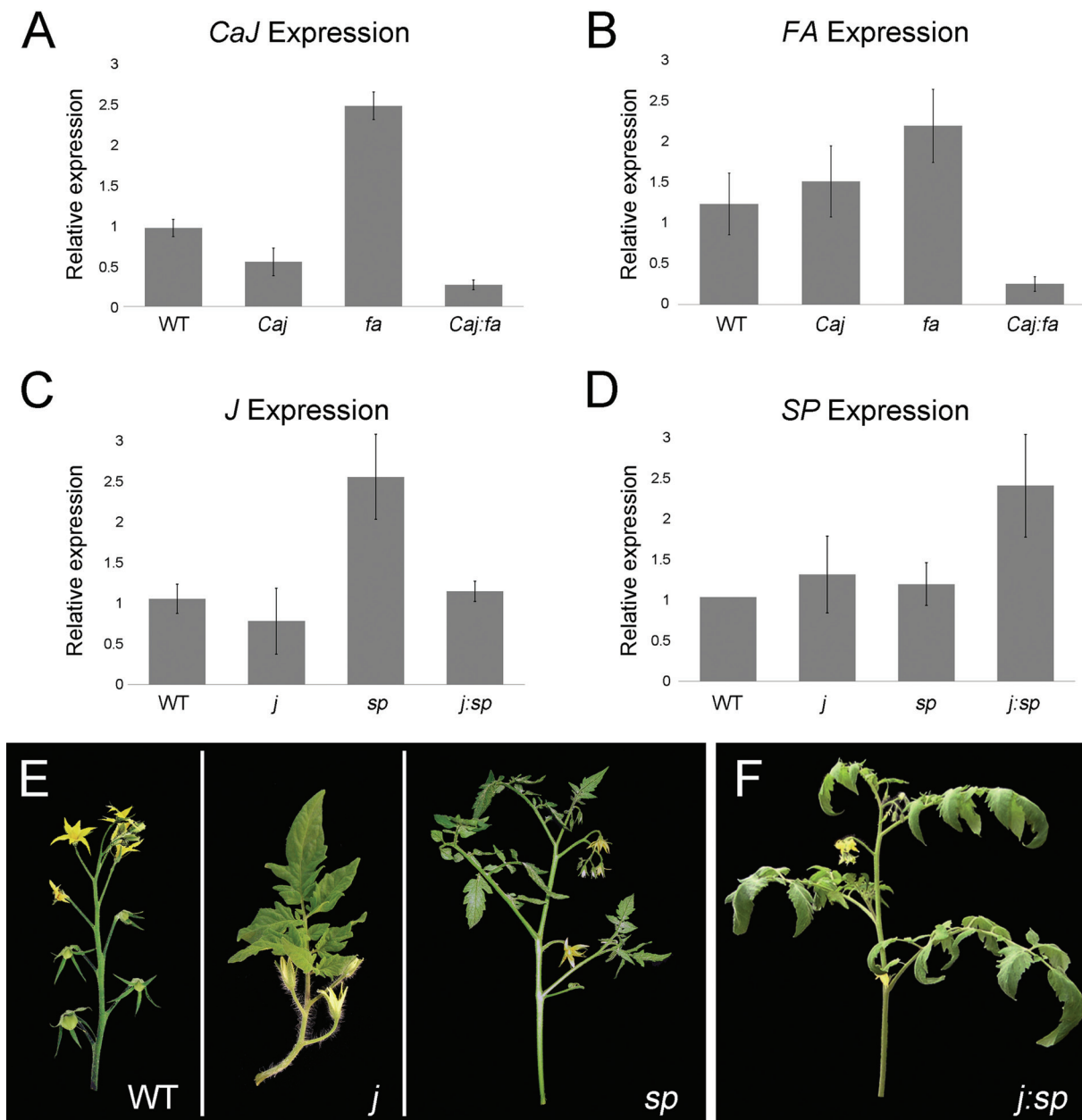


Fig. 5. Expression patterns of *CaJ*, *FA* and their tomato homologues in sympodial apices. (A, B) Expression levels of *CaJ* and *FA* in sympodial apices of wild-type, *Caj* (*E-252*), *fa*, and *Caj:fa* pepper. (C, D) Expression levels of *JOINTLESS* (*J*) and *SP* in sympodial apices of wild-type, *j*, *sp*, and *j:sp* tomato. (E) Inflorescences and sympodiums of tomato genotypes: left, wild type; centre, *j* forming a few flowers followed by leaves; right, *sp* showing reduction of leaf number between inflorescences. (F) Inflorescence and sympodium of tomato double mutant *j:sp*. The sympodial shoot and inflorescence are similar to *sp*.

including the apical, sympodial, and floral meristems, can independently delay their termination. The enhanced precocious sympodial termination in *Caj:CaBl* compared to *CaBl* is similar to the homologous tomato double mutant that produces a single terminal flower (Szymkowiak and Irish, 2006). The synergistic action of *CaJ* and *CaBL* in controlling flowering time and sympodial development as inferred from the double mutant indicate that the two genes interact with each other in controlling pepper architecture.

The stage-specific effects of mutations in genes such as *SP* and *TERMINATING FLOWER* (*TMF*) on termination of the

sympodial shoot and primary shoot of tomato, respectively, led to the conclusion that two flowering systems exist in tomato: one for the primary shoot and a second for the sympodial shoot (Lifschitz and Eshed, 2006). The late-flowering mutants of pepper (*CaBl*, *Caj*, and *E-172-1*) do not show such stage-specific phenotypes of the two shoot systems, as both the primary and sympodial shoots are late flowering. Similarly, the early termination phenotype of *fa* is observed in both shoot systems. Although this coordination may be lost in other mutants, it is possible that in pepper, flowering regulation of the two shoot systems is more tightly coordinated than in tomato.

Role of *CaJ* in controlling sympodial development

The main effect of *Caj* on sympodial development is the sporadic addition of leaves within the sympodial units. In contrast, tomato *j* has been mostly associated with lack of abscission zone on the flower pedicel and to a lesser extent with reversion of the inflorescence into vegetative growth (Szymkowiak and Irish, 2006). According to Emery and Munger (1970), an increased number of leaves between inflorescences was also observed in tomato *j*; however, other studies indicate no change in the sympodial growth pattern between the wild type and *j* (Thouet et al., 2012). Since wild-type pepper lacks an abscission zone on the flower pedicel, the effect of *CaJ* on abscission zone development in the pedicel cannot be assessed.

Increased expression of *CaJ* in the *fa* mutant and strong epistasis of *Caj* over *fa* in the *Caj:fa* double mutant indicated that *FA*, the pepper orthologue of tomato *SP*, is upstream of *CaJ* in the pathway controlling sympodial development and inhibits it (Fig. 4F, 5A). In tomato, inactivation of *SP* results in early termination of the sympodial shoot but with no change in flowering time of the primary shoot (Pnueli et al., 1988). In pepper, *fa* is characterized by early flowering of the primary shoot and compact sympodial units (Elitzur et al., 2009). Therefore, in both tomato and pepper, *SP* and *FA* have functions opposite to those of *J* and *CaJ*, respectively, by repressing the termination of shoot apices. However, while in pepper, *Caj* is epistatic to *fa* in all aspects of primary and sympodial shoot development, the relationship between the two homologous mutations in tomato is more complex and depends on the genetic background. *sp* has been reported to be epistatic to *j* with respect to inflorescence development (Rick and Butler, 1956; Szymkowiak and Irish, 2006), while late flowering of *sp:j* was similar to *j*.

Role of *CaJ* in flower development

Instead of the single flower that develops in wild-type pepper, pedicels developing from the *Caj* mutant often carry multiple (two or three) flowers and vegetative growth is evident on the flower pedicel (Fig. 1F). The appearance of multiple flowers and leaves on the flower pedicel indicates that the pepper's solitary flower has a cryptic inflorescence identity and that this inflorescence has a sympodial nature, similar to that of tomato. Support for this conclusion comes from the indeterminate flower phenotype of pepper *Ca-an* mutant (Lippman et al., 2008) and the double mutant *Caj:Ca-an* (Fig. 4D). A model suggesting the rate of meristem maturation as a key factor in the regulation of inflorescence architecture was proposed by Park et al. (2012). According to that model, delayed meristem maturation promotes branching inflorescences, such as the *compound inflorescence (s)* mutant in tomato (Lippman et al., 2008), while rapid maturation of the inflorescence meristem leads to formation of a single flower in species such as pepper. Deceleration of inflorescence meristem maturation in the *Caj* background leads to the formation of a sympodial shoot-like structure composed of leaves and flowers. Therefore, similar to the function of *J* in repressing sympodial identity of the inflorescence in tomato, *CaJ* has evolved to suppress sympodial inflorescence growth in pepper and to promote early maturation of the inflorescence meristem to produce a single flower.

In conclusion, *CaJ* functions as a repressor of vegetative growth in all shoot meristems. In agreement with this function, its expression is observed in all shoot meristems. While the pepper gene has functions similar to its tomato orthologue, the effect in pepper on controlling flowering time and sympodial development is more pronounced than in tomato. Moreover, both genes also evolved to have specific functions. While *J* is involved in abscission-zone formation in the flower pedicel of tomato, *CaJ* maintains single-flower formation in pepper. This study demonstrates that induced mutagenesis and identification of multiple mutant alleles is an effective method to generate variation in shoot architecture and study the function of the major genes controlling this trait. Such an approach is particularly useful in species such as pepper for which transformation is inefficient as a tool to study gene function.

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