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ORIGINAL PAPER

CaAP2 transcription factor is a candidate gene for a flowering repressor and a candidate for controlling natural variation of flowering time in *Capsicum annuum*

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Abstract

Key message **The *APETALA2* transcription factor homolog *CaAP2* is a candidate gene for a flowering repressor in pepper, as revealed by induced-mutation phenotype, and a candidate underlying a major QTL controlling natural variation in flowering time.**

Abstract To decipher the genetic control of transition to flowering in pepper (*Capsicum* spp.) and determine the extent of gene function conservation compared to model species, we isolated and characterized several ethyl methanesulfonate (EMS)-induced mutants that vary in their flowering time compared to the wild type. In the present study, we report on the isolation of an early-flowering mutant that flowers after four leaves on the primary stem compared to nine leaves in the wild-type 'Maor'. By genetic mapping and sequencing of putative candidate genes linked to the mutant phenotype, we identified a member of the *APETALA2* (*AP2*) transcription factor family, *CaAP2*, which was disrupted in the early-flowering mutant. *CaAP2* is a likely ortholog of *AP2* that functions as a repressor of flowering in *Arabidopsis*. To test whether *CaAP2* has an effect on controlling natural variation in the transition to flowering in pepper, we performed QTL mapping for flowering time in a cross between early and late-flowering *C. annuum* accessions. We identified a major QTL in a region of chromosome 2 in which *CaAP2* was the most significant marker, explaining 52 % of the phenotypic variation of the trait. Sequence comparison of the *CaAP2* open reading frames in the two parents used for QTL mapping did not reveal significant variation. In contrast, significant differences in expression level of *CaAP2* were detected between near-isogenic lines that differ for the flowering time QTL, supporting the putative function of *CaAP2* as a major repressor of flowering in pepper.

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Introduction

Flowering time is a major adaptive trait of crop plants; it has an important role during domestication and subsequent selection toward growth optimization in different environments and in the expansion of crop production to new climatic zones (Zuellig et al. 2014). For example, a change in the response to photoperiod sensitivity of flowering allowed expansion of crops such as corn, rice and cotton that were domesticated in tropical regions to temperate regions (Hung et al. 2012; Lu et al. 2012; Olsen and Wendel 2013). Similarly, a change in vernalization requirements of the temperate cereals wheat and barley had a key role in the development of spring varieties of these crops (Golovnina et al. 2010; Olsen and Wendel 2013).

Flowering time is under the complex control of endogenous and environmental signals such as plant age,

hormones, day length and temperature (Srikanth and Schmid 2011). *Arabidopsis thaliana* is the most explored species for the genetic control of flowering. Several pathways, quantitative trait loci (QTLs) and numerous genes have been identified in *Arabidopsis* that control the transition to flowering in artificial mutants and natural populations (Gul Khan et al. 2014). Many of the pathways and genes controlling flowering time are conserved across species. However, diversification of gene functions and interactions varies at the species level. Furthermore, some flowering genes in monocots do not have functional equivalents in dicots (Shrestha et al. 2014), emphasizing the need to study the species-specific regulation of this fundamental trait of crop plants.

Regulation of flowering time in *Arabidopsis* is orchestrated by a complex network of flowering-promoter and suppressor genes. The key flowering promoter is *FLOWERING LOCUS T* (*FT*), which is considered to be florigen. Florigen is produced in the leaf and the signal is transported to the shoot apical meristem (SAM) where flowering is induced (Corbesier et al. 2007). Floral repressors are essential to preventing premature flowering, via direct or indirect interactions with *FT* and other flowering promoters (Yant et al. 2009). One major repressor is the MADS-domain transcription factor *FLOWERING LOCUS C* (*FLC*) that binds to several genes involved in the transition to flowering, among them *FT* and the flowering promoter *SUPPRESSOR OF OVEREXPRESSION OF* CONSTANS*1* (*SOC1*) and inhibits their activation (Deng et al. 2011). A second MADS-domain gene, *SHORT VEGETATIVE PHASE* (*SVP*), acts together with *FLC* to repress flowering (Li et al. 2008). A second class of transcription factors that acts as flowering repressors is a clade of six *APETALA2* (*AP2*)-domain partially redundant genes that are targets of *microRNA172* (*miR172*) (Aukerman and Sakai 2003; Yant et al. 2010). This clade consists of three *TARGET OF EAT* genes (*TOE1*, *TOE2* and *TOE3*), *SCHLAFMUTZE* (*SMZ*), *SCHNARCHZAPFEN* (SNZ), and *AP2*. Genome-wide direct-binding assays and expression analyses have indicated that *AP2* acts as a bifunctional transcription factor that regulates flowering by both suppression of flowering activators and activation of flowering suppressors (Yant et al. 2010).

In the Solanaceae family, tomato is the best characterized species for genetic control of transition to flowering (reviewed by Samach and Lotan 2007). However, the extent of conservation of genes controlling this trait in additional crop species in the family has been little studied. To dissect the genetic control of transition to flowering and shoot architecture in pepper (*Capsicum* spp.), we screened an EMS-mutagenized population for alterations in these traits. This screen enabled identifying several late-flowering mutants, including *CaJOINTLESS*, *CaBLIND* and *CaS* (Jeifetz et al. 2011; Cohen et al. 2012, 2014). We further found that the compact early-flowering mutant *fasciculate* is controlled by the pepper homolog of *Arabidopsis TERMINAL FLOWER1* (*TFL1*, Elitzur et al. 2009). These initial studies already indicate the diversification of pepper-specific genes in controlling flowering compared to *Arabidopsis*. For example, while *CaJOINTLESS* is a major flowering activator in pepper, *SVP*, its *Arabidopsis* homolog is a major flowering repressor (Cohen et al. 2012). Furthermore, while *CaS* is required for flower formation in pepper, its closest *Arabidopsis* homolog functions in embryonic patterning but does not affect flowering (Cohen et al. 2014).

In the present study, we describe a new early-flowering mutation in pepper, *E-62*, and provide evidence for impairment of a member of the *AP2* gene family (*CaAP2*) in this mutant. Furthermore, we tested whether this gene can account for controlling natural variation of flowering time by performing a QTL study for this trait in a cross between parents that exhibit extreme variations for flowering time. Our QTL mapping study indicated that the genomic region containing *CaAP2* has a highly significant effect on flowering time. Furthermore, the expression level of this gene is significantly higher in a late-flowering near-isogenic line (NIL) than in an early-flowering NIL differing for *CaAP2* alleles. Our data, described in the present manuscript using induced and natural variation for flowering time, collectively support our hypothesis that *CaAP2* is a likely candidate for being a major flowering time regulator in pepper.

Materials and methods

Plant material

The early-flowering mutant *E-62* was isolated from an EMS-mutagenized population with *Capsicum annuum* cv. Maor as the wild-type parent (Paran et al. 2007). The mutant was crossed to 'Maor', and F_3 plants homozygous for the mutation were used for the experiments described in the present study. For molecular mapping, an F_2 segregating population was generated by crossing *E-62* to *Capsicum frutescens* BG 2816. For QTL analysis of flowering time (measured as the number of leaves on the primary stem until first flower), an F_2 segregating population consisting of 230 progeny was generated by crossing early-flowering *C. annuum* accession PI 527325 (hereafter USDA-early) and the late-flowering *C. annuum* var. *glabriusculum* wild accession PI 511887 (hereafter USDA-late), both obtained from the USDA pepper collection in Griffin, GA, USA. For QTL verification, BC_2F_1 and BC_2F_2 populations were created using USDA-early as the recurrent parent. A BC_2F_2 population consisting of 142 individuals resulted from selfing a single BC_2F_1 plant heterozygous for

the most significant marker at the QTL detected in the F_2 generation. QTL-NILs were prepared by marker-assisted selection and a backcrossing program using USDA-early as the recurrent parent and USDA-late as the donor parent. QTL-NILs were derived from BC_3F_2 individuals that were heterozygous at the most significant marker of the QTL and subsequent selfing to obtain homozygous BC_3F_4 plants for the recurrent and donor alleles.

Mapping and QTL analyses

To map the gene governing the *E-62* phenotype, the bulked segregant analysis approach was used (Michelmore et al. 1991). Two bulks of DNA composed of 15 individuals each from mutant and wild-type individuals of the F_2 segregating population were constructed and screened with COSII markers (http://www.sgn.cornell.edu) distributed throughout the genome. After initial assignment of the mutation to chromosome 2, a larger F_2 population consisting of 600 progeny was phenotyped and 125 mutant individuals were used for fine mapping of the gene using markers from chromosome 2.

For QTL mapping, an initial genetic map was constructed using proprietary single-nucleotide polymorphism (SNP) markers in chromosome 2 (Nunhems Netherlands B.V). The map was subsequently enriched with public COSII markers that allowed anchoring to previously published maps. A genetic map was constructed using MAPMAKER software (Lander et al. 1987). Map distances were computed with the Kosambi mapping function. Interval mapping QTL analyses in the segregating F_2 population were performed with QGENE v. 3.04 software (Nelson 1997). Significance threshold level (LOD 3.2) for QTL detection was computed by permutation tests with 1000 iterations at $P < 0.01$. Single-marker QTL analysis in the BC_2F_2 generation was performed by ANOVA using JMP v. 10 software (SAS Institute Inc., Cary, NC, USA).

Isolation of *CaAP2*

To clone *CaAP2*, we screened the UC Davis transcriptome database with the tomato *AP2* homolog Solyc02g093150 and identified *C. annuum* MGMT contig749 (GenBank accession JW050993; Ashrafi et al. 2012) as containing the complete open reading frame (ORF) of the homologous pepper gene. This sequence was used as a template to design primers AP2-5′F and AP2-R3 (Table S1) that flank the ORF for sequencing *CaAP2* from the parents used in this study (GenBank accessions numbers KM594389–KM594391). The ORF sequences were translated and used for multiple alignments by the web-based version of Clustal W (http://www.ebi.ac.uk/services). Screening for *E-62* mutants in subsequent experiments was performed by developing a CAPS (cleaved amplified polymorphic sequence) marker using the primers AP2-F1 and AP2-R2 (Table S1) followed by restriction digestion with the enzyme *Hpy*188I that enables distinguishing the mutant from wild-type alleles.

Gene-expression analysis

Total RNA was extracted from shoot apices using GeneElute Mammalian Total RNA Extraction Miniprep kit (Sigma) followed by DNaseI treatment (Sigma). Total RNA (400 ng) was used for first-strand cDNA synthesis by reverse transcription (RT) PCR using the PrimeScript RT Reagent kit (Takara). For real-time quantitative PCR (qRT-PCR), five 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 (day length 10–11 h). PCR amplification was performed using the primers AP2-RT-F and AP2-RT-R (Table S1). Amplified products were detected using SYBR Premix Ex Taq II (Takara) in a Rotor-Gene 6000 thermal cycler (Corbett). Results were analyzed using Rotor-Gene 6000 Series software 1.7 (Corbett). The relative expression levels of the gene were normalized against *CaUBIQUITIN* (DQ975458.1) using the primers UBQ-qRTF and UBQ-qRTR. Digital expression data in RPKM (reads per kilobase of transcript per million mapped reads) for *CaAP2* in 'Maor' were derived from transcriptome profiling data of SAMs at four sequential developmental stages—vegetative, transition, floral and sympodial—in two repeats as previously described (Park et al. 2012). Raw data were generated by M. Schatz and Z. Lippman, Cold Spring Harbor Laboratory and deposited in Sol Genomics Network (http://www.sgn.cornell.edu).

Scanning electron microscopy (SEM)

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

Phylogenetic analysis

Multiple sequence alignments were performed with a web-based version of ClustalW (http://www.ebi.ac.uk/tools/clustalw/) using the default settings. The phylogenetic tree was calculated by neighbor-joining method and bootstrap analysis with 1000 replicates via MEGA4 software (http://www.megasoftware.net/mega4/mega.html). The tree was calculated from alignments of a 171-amino acid region flanking the two AP2 domains of the protein (Fig. S1). Genes and accession numbers were as follows; pepper: CA02g14540, CA02g01830,

Table 1 Growth parameters of ‘Maor’ and *E-62*

	No. of leaves at first flower	Height at first flower (cm)	Length of first internode (cm)	1st leaf area (cm^2)	Fruit weight (g)
Maor	8.6 ± 0.5***	17.8 ± 1.3***	3.7 ± 0.9***	34 ± 8.6**	97.4 ± 12.9***
E-62	4 ± 0	5.3 ± 0.7	1.4 ± 0.4	20.1 ± 9.2	54.7 ± 13

Asterisks indicate significant difference between ‘Maor’ and *E-62* by Student’s *t* test (*** $P < 0.0001$; ** $P < 0.001$)

CA04g0813, CA11g14070, CA00g85250; tomato: SlAP2a (ACD62792), SlAP2b (HQ586952), SlAP2c (HQ586951), SlAP2d (HQ586953), SlAP2e (HQ586954); potato: StRAP1 (CAR92295.1); petunia: PHAp2A (AAD39439); arabidopsis: AtAP2 (AAC13770), AtSNZ (Q6PV67), AtSMZ (Q6PV68), AtTOE1 (Q9SK03), AtTOE2 (Q9LVG2), AtTOE3 (Q9FH95).

Results

Characterization of wild type and mutant

The early-flowering monogenic recessive mutant *E-62* was isolated from an EMS-mutagenized population of cv. Maor. Whereas ‘Maor’ flowers after 8.6 ± 0.5 leaves on the primary stem, *E-62* flowers after 4 ± 0 leaves (Table 1; Fig. 1a, b). The sympodial shoot structure of *E-62* has the typical dichasially forked shoot of wild-type pepper (Cohen et al. 2014). However, shoot and fruit sizes are smaller than in ‘Maor’ (Table 1; Fig. 1c). No changes in flower structure were observed in *E-62* compared to ‘Maor’. To determine the SAM developmental stage at which initiation of flowering occurs in *E-62*, we examined a series of apices by SEM at different stages. In ‘Maor’, at the 2-leaf stage, the SAM is vegetative. Initiation of flower formation is observed at the stage of four expanding leaves (defined as the number of leaves on the primary stem that are at least 3 cm long), while a fully differentiated flower is observed at the 6-expanding-leaf stage and sympodial meristems are observed at the flank of the apical flower (Fig. 2). In *E-62*, the transition to flowering occurs sooner, with full flower differentiation at the 2-leaf stage.

Fig. 1 Pictures of the wild-type cv. Maor and the early-flowering mutant *E-62*. **a** ‘Maor’ flowers after nine leaves on the primary stem. **b** *E-62* flowers after four leaves on the primary stem. **c** ‘Maor’ and *E-62* after fruit set

CaAP2 is disrupted in *E-62*

We employed bulked segregant analysis to map *E-62* in the pepper genome. *E-62* was assigned to the distal end of chromosome 2 within a 5-cm interval between the COSII markers C2_At5g67370 and C2_At4g37280 (Wu et al. 2009). The mutation was subsequently fine-mapped using 125 mutant individuals selected from an F_2 population of 600 progeny to a region of approximately 350 kb between Solyc02g092910 and Solyc02g093300 in the tomato genome. We initially examined the annotation of this region in tomato, which contains 44 genes (Table S2). Upon publication of the pepper genome (Kim et al. 2014), we determined that the pepper gene content in this region is similar to that of tomato (Table S2). We identified four genes that might function in the regulation of flowering based on prior knowledge in *Arabidopsis* (Solyc02g092910—chromatin binding protein, Solyc02g093150—AP2-like ethylene-responsive transcription factor, Solyc02g093190—MEDEA and Solyc02g093200—histone-lysine *N*-methyltransferase MEDEA). We sequenced the ORFs of these genes in ‘Maor’ and *E-62* and found that the only gene that differed in sequence between the wild type and mutant was the pepper ortholog of Solyc02g093150 (CA02g14540). Members of the *AP2* gene family suppress flowering time

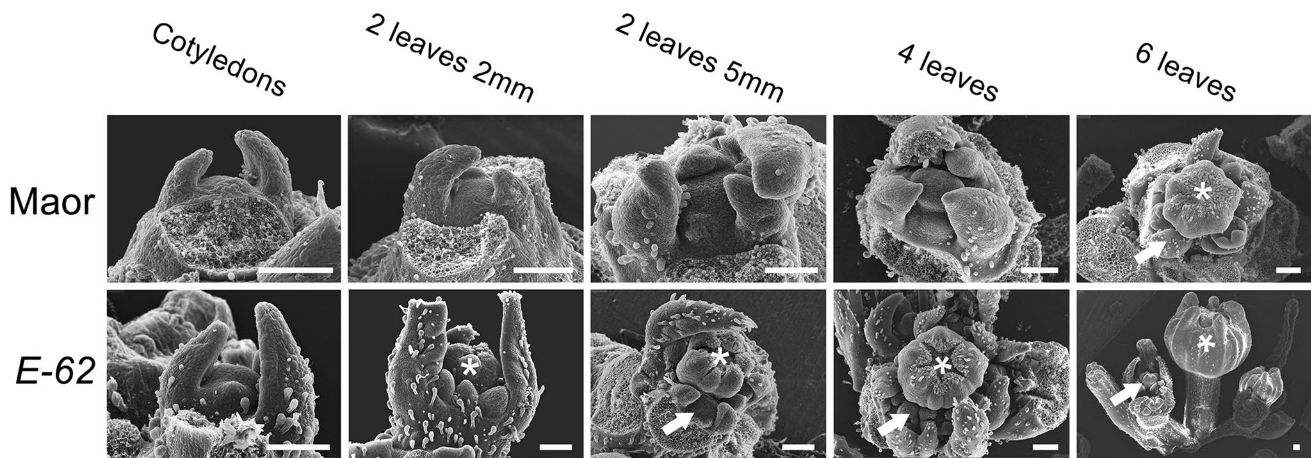

Fig. 2 Scanning electron micrographs of developing SAM of 'Maor' and *E-62*. In 'Maor' and *E-62*, flower differentiation starts at the 4-leaf and 2-leaf stage, respectively. The terminal flower is marked by an *asterisk* and sympodial meristems composed of two leaves and a flower primordium are marked with *white arrows*. *Bars*, 200 µm

in *Arabidopsis* (Yant et al. 2010), sharing a similar function with the gene governing the *E-62* mutation. Sequence comparison between 'Maor' and *E-62* revealed a single nucleotide change from G in the wild-type allele to A in the mutant allele at nucleotide 525 of the ORF. This SNP led to the creation of a premature stop codon and a putative truncated protein of 174 amino acids compared to 524 amino acids in the intact protein (Fig. S1). A CAPS marker (Table S1) developed on the basis of the SNP allowed following the mutation in a segregating population of 'Maor' × *E-62* and indicated complete linkage with the phenotype.

Observing the RNA-Seq expression data in various tissues for CM334 used to sequence the pepper genome (Kim et al. 2014), we found *CaAP2* expression in all of the examined tissues (leaf, root, stem, fruit pericarp). We further measured its expression pattern in the SAM of 'Maor' at different stages of development, from the vegetative to sympodial meristem stage. *CaAP2* was highly expressed at the vegetative meristem stage and, as expected based on its putative function as a flowering repressor, its expression level was dramatically reduced during the transition to flowering and flower formation (Fig. 3a). A phylogenetic tree of *AP2*-related sequences from *Arabidopsis* and Solanaceae species indicated that *CaAP2* is most closely related to tomato *SlAP2c* (Fig. 3b) with unknown function.

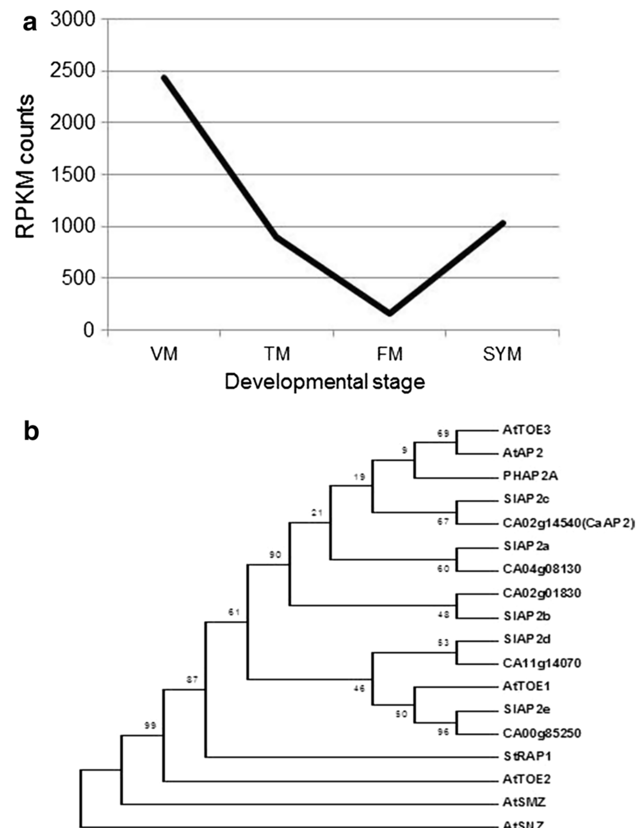

Fig. 3 Expression level of *CaAP2* and phylogenetic tree of *CaAP2*-related genes. **a** Digital gene-expression level of *CaAP2* presented as RPKM values in SAM of 'Maor' at sequential meristem developmental stages. *VM* vegetative meristem, *TM* transition meristem, *FM* floral meristem, *SYM* sympodial meristem. **b** Phylogenetic tree of CaAP2 and its homologs from pepper, tomato, petunia, and *Arabidopsis*. Numbers indicate percentage bootstrap support for each branch (1000 replicates)

A major flowering time QTL is mapped in the *CaAP2* region

To test whether *CaAP2* has a role in regulating natural variation in flowering in pepper, we conducted a QTL mapping study in a cross of early-flowering *C. annuum*

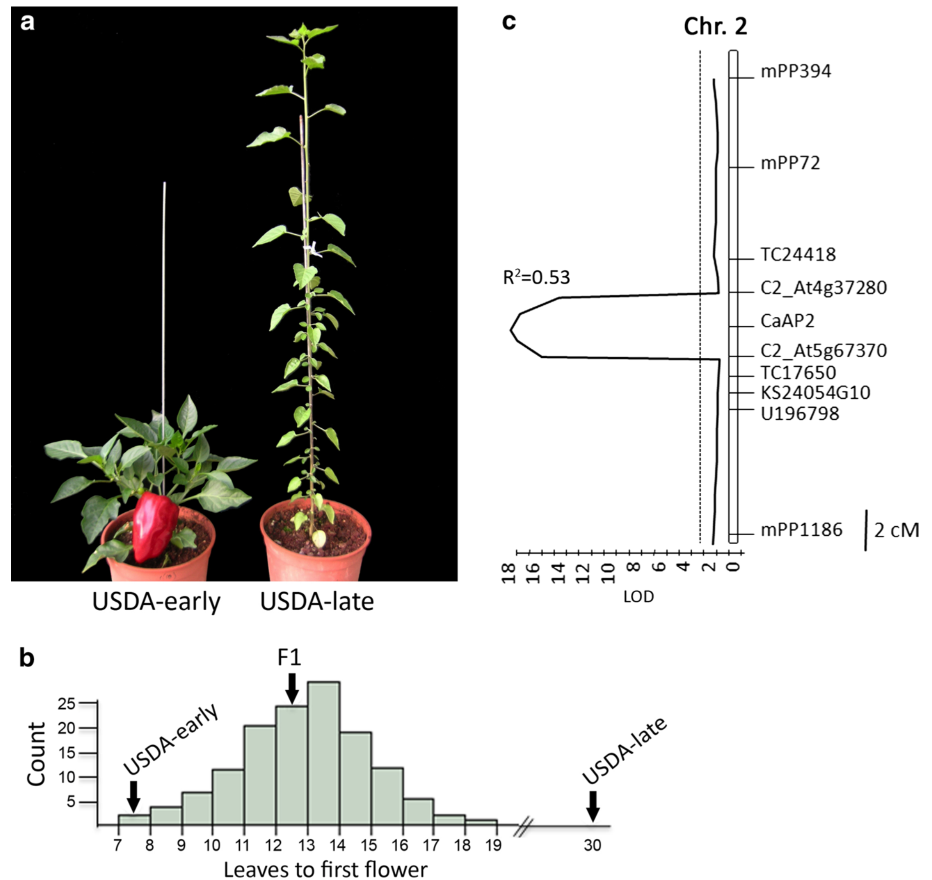

Fig. 4 QTL mapping of flowering time in a cross of USDA-early and USDA-late. **a** Pictures of the early- and late-flowering parents used for QTL mapping. **b** Distribution of flowering time in the F_2 population. **c** Interval QTL mapping of flowering time in a region containing *CaAP2* in chromosome 2

Table 2 QTL parameters for flowering time in F_2 and BC_2F_2 generations of USDA-early × USDA-late

Generation	Marker	Means			R^2 (%)	*P*
		AA	AB	BB		
F_2	*CaAP2*	9.7 ± 1.4	13.2 ± 1.2	15.2 ± 1.5	52	<0.0001
BC_2F_2	*CaAP2*	8.3 ± 1	11.3 ± 1	13.0 ± 1	50	<0.0001

Flowering time was defined as number of leaves on the primary stem until first flower. QTL analysis in the F_2 was performed by interval mapping and in the BC_2F_2 by single-marker analysis

AA homozygous for USDA-early allele, *AB* heterozygous, *BB* homozygous for USDA-late allele

USDA-early (flowers after 7.8 ± 0.4 leaves on the primary stem) and the late-flowering wild pepper USDA-late that flowers after 30 ± 2 leaves on the primary stem (Fig. 4a). The continuous segregation of flowering time in the F_2 population indicated quantitative inheritance of this trait (Fig. 4b). The recovery of F_2 individuals with flowering time similar to USDA-early but not to USDA-late, and the flowering time of the F_1 generation (12 ± 1 leaves on the primary stem), indicated partial dominance of early flowering. We tested a region of 30 cM that flanks *CaAP2* for the presence of a QTL for flowering time. The most significant marker at the region (LOD 18) was *CaAP2*, explaining 52 % of the phenotypic variation of the trait (Fig. 4c; Table 2). The location and effect of the QTL was subsequently verified in the BC_2F_2 generation (Table 2).

To test the effect of *CaAP2* on flowering time in a uniform genetic background, we constructed QTL-NILs for the locus. We selected two individuals in the BC_3F_3 generation from the cross USDA-early × USDA-late that differed for flowering time and for *CaAP2* alleles. Plant BC_3F_3-34 flowered after eight leaves on the primary stem and had the USDA-early allele at *CaAP2*, whereas plant BC_3F_3-37 flowered after 14 leaves on the primary stem and had the USDA-late allele at *CaAP2*. These plants were self-pollinated and the means of BC_3F_4-34 (hereafter early NIL) and BC_3F_4-37 (hereafter late NIL) families were 8.6 ± 1 and 13.6 ± 1 leaves to first flower, respectively (Fig. 5a).

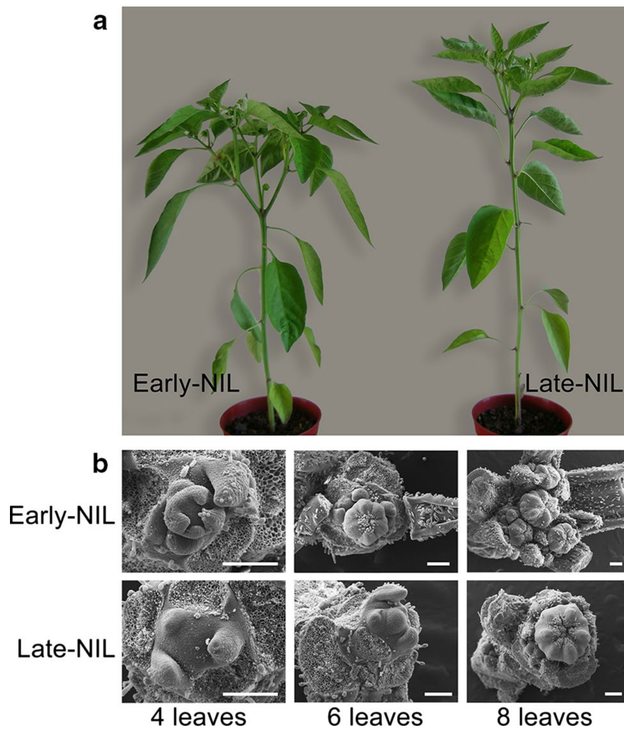

Fig. 5 Near-isogenic lines (NILs) for *CaAP2* derived from the cross of USDA-early and USDA-late. **a** Pictures of the early- and late-flowering *CaAP2*-NILs. **b** Scanning electron micrographs of developing SAM of *CaAP2*-NILs. Initiation of flower differentiation occurs at the 4-expanding-leaf and 6-expanding-leaf stage in early- and late-flowering NILs, respectively. *Bars*, 200 µm

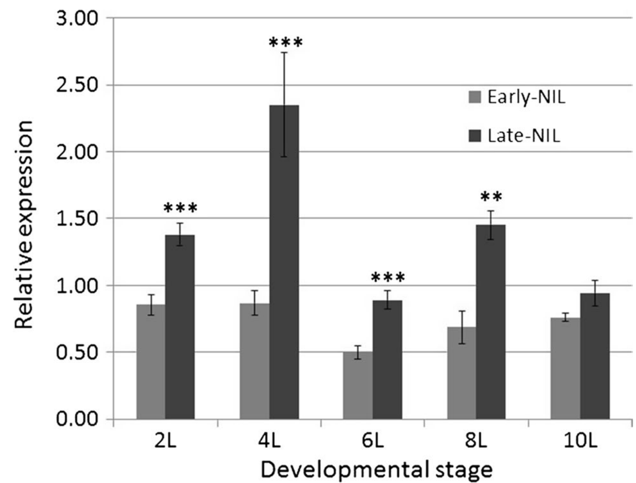

Fig. 6 Quantitative RT-PCR expression of *CaAP2* in SAM of *CaAP2* near-isogenic lines (NILs) at the 2-, 4-, 6-, 8-, and 10-expanding-leaf stage (L). Data for each group are means of five independent replicates ±SE. *Asterisks* indicate significant difference (***$P < 0.001$, **$P < 0.01$) between *CaAP2*-NILs determined by Student's *t* test

The fixation of flowering time within the BC_3F_4 families and the similar phenotypic values of the BC_3F_3 and BC_3F_4 generations indicated that no additional QTLs segregated in these families. In the early NIL, initiation of flower formation was observed in the SAM at the stage of four expanding leaves, while the fully differentiated flower was observed at the 6-expanding-leaf stage. In the late NIL, initiation of flower formation was observed in the SAM at the 6-expanding-leaf stage, while the fully differentiated flower was observed at the 8-expanding-leaf stage (Fig. 5b).

To further substantiate the involvement of *CaAP2* in controlling flowering time variation in the USDA-early × USDA-late cross, we compared the sequence of the ORF between these two parents (Fig. S1). Two non-polar amino acid substitutions were observed between the two parents as well as two small deletions of two amino acids each in USDA-late that did not change the reading frame of the protein. We further determined the expression pattern of *CaAP2* in the SAM of both NILs at different stages of development, from vegetative meristem to reproductive transition (Fig. 6). At all stages, the level of expression of *CaAP2* was higher in the late than in the early NIL, as expected based on the gene's function as a flowering suppressor. The largest difference in expression was observed at the 4-expanding-leaf stage, which corresponds to the transition to flowering in the early NIL and a vegetative meristem in the late NIL (Fig. 5b).

Discussion

CaAP2 is a candidate for a flowering repressor in pepper

We report the isolation of an early-flowering mutant which enabled the identification of a member of the *AP2* transcription factor family, *CaAP2*, as a likely flowering repressor in pepper. Because of the reduction of shoot and fruit size associated with early flowering and the extreme flowering time phenotype, this mutation is not likely to be utilized in pepper breeding except for types that are adapted for a very short season. While we provide strong genetic supporting evidences for the putative function of *CaAP2* as a flowering repressor, we cannot exclude the possibility that a different tightly linked gene may account for the phenotype. A proof for the function of *CaAP2* will require additional experiments such as identification of additional independent allelic mutants or complementation of the mutant phenotype by transformation.

The dramatic effect on flowering time conferred by a lesion in *CaAP2* indicates little redundancy of this gene with other *AP2*-like members or other genes in pepper; this is in contrast to the significant redundancy observed among the *AP2* gene family members in controlling flowering time in *Arabidopsis* (Mathieu et al. 2009; Yant et al. 2010). Compared to *Arabidopsis*, *CaAP2* is most closely related

to *AP2* and *TOE3* (Fig. 3b), both of which act as flowering suppressors and control flower patterning (Bowman et al. 1989; Yant et al. 2010; Jung et al. 2014). In *Arabidopsis*, *AP2* acts primarily in the SAM by repressing the flowering-promoter genes *AGAMOUS* (*AG*) and *SOC1*, while not affecting the expression of *FT* in the leaf (Yant et al. 2010). *AP2* has wide pleiotropic effects in *Arabidopsis*, affecting flower development, seed mass and fruit development (Bowman et al. 1989; Ohto et al. 2005; Ripoll et al. 2011). In pepper *E-62*, we observed a reduction in shoot organ size but we did not observe changes in flower organ structure or seed mass. This indicates that apart from regulation of transition to flowering, diversification of gene function has occurred between pepper and *Arabidopsis*, or other genes redundantly control floral patterning.

In recent years, we have isolated several flowering-promoter genes in pepper by screening an EMS-mutagenized population for alterations in flowering time and sympodial growth. These have included *CaJOINTLESS*, *CaBLIND*, *CaS* and an additional late-flowering mutant *E-172* for which the underlying gene has not been identified (Paran et al. 2007; Jeifetz et al. 2011; Cohen et al. 2012, 2014). The only known flowering repressor in pepper is *FASCICULATE*, the homolog of *Arabidopsis TFL1* that was isolated as a spontaneous natural mutant (Elitzur et al. 2009). The primary effect of *FASCICULATE* is control of sympodial shoot structure, while its effect on flowering time is minor. Screening about 4000 M2 families for changes in flowering time enabled the detection of a single early-flowering mutant—*E-62*, described in the present study. The low frequency of detection of early-flowering mutants might be explained by the elimination of flowering-suppressor genes in elite lines such as 'Maor' during the long history of pepper breeding. This situation is similar to tomato, in which most flowering mutants are late flowering and only a few, such as *terminating flower*, are early flowering (MacAlister et al. 2012).

The function of *AP*-like genes as flowering suppressors has been well established in the monopodial species *Arabidopsis* (Aukerman and Sakai, 2003; Yant et al. 2010). In another monopodial species, maize, a major flowering time QTL at the *VEGETATIVE TO GENERATIVE TRANSITION 1* (*VGT1*) locus was determined to be associated with polymorphism at a noncoding region upstream of an *AP2* homolog (Salvi et al. 2007). However, the extent of conservation of the *AP2* gene family in controlling flowering time in plants that exhibit sympodial shoot structure is not known. The Solanaceae family includes many important plant species with sympodial shoot structure, such as tomato, petunia and pepper. In tomato, five *AP2* paralogs are known that belong to the euAP2 lineage, the closest homolog to *Arabidopsis AP2* being *SlAP2a* which functions in the regulation of fruit ripening (Chung et al. 2010; Karlova et al. 2011). A change in flowering time was not reported in transgenic silenced *SlAP2a* plants. Mutations in other tomato *AP2* genes are not yet known. High expression of *SlAP2c*, the closest tomato homolog of pepper *CaAP2* in flowers, suggests a likely role for this latter gene in flower development (Karlova et al. 2011). Transposon mutagenesis in the putative *Petunia* ortholog of *CaAP2*, *PHAP2A* (Fig. 3b), had no recognizable phenotypic effect (Maes et al. 2001). However, a possible function for the *AP2* family in flowering time regulation in the Solanaceae was reported in potato (Martin et al. 2009). In that organism, *miR172* promoted flowering by overexpression as well as the induction of tuberization, and an *AP2*-like target gene of *miR172*, *RAP1*, was identified and proposed to be a repressor of these processes. This gene is most closely related to the *Arabidopsis* flowering repressors *TOE2*, *SMZ* and *SNZ* in the phylogenetic tree (Fig. 3b).

CaAP2 is a candidate for underlying a major flowering time QTL

We further report on the identification of a major flowering time QTL which resides in the genomic region containing *CaAP2*. At this point, we cannot prove that *CaAP2* is the gene that underlies this QTL. However, based on analysis of the mutant *E-62*, we provide strong indication of this gene's function as a flowering repressor. Furthermore, its expression in the SAM is at a significantly higher level in a late-flowering NIL compared to an early-flowering control. Additional work, such as higher resolution mapping and complementation analysis by stable or transient transformation will be required to prove that *CaAP2* underlies the flowering time QTL.

While in *E-62*, putative loss of function due to a premature stop codon is the likely cause of the mutant phenotype, sequence comparison between *CaAP2* alleles of the parents used for QTL mapping did not reveal clear causative polymorphism in the coding region. We cannot rule out the possibility that the sequence variations between USDA-early and USDA-late may contribute to change in the gene activity or in its expression pattern. However, because none of these changes occurred in the AP2 domains or the miR172-binding site, we assume that no major changes in the activity of the protein are expected. Our hypothesis is that *cis*-regulatory polymorphisms cause changes in the gene's expression and underlie the QTL. While we were able to demonstrate significant expression differences between the two *CaAP2* alleles of the late- and early-flowering parents, we were not able to associate this difference with specific sequence variation in the promoter region. However, sequence divergence in the promoter is very possible because of the failure to PCR amplify a region of ~1200 bp upstream of the start codon (nucleotides 619421–620626 in PGAv.1.5.scaffold370; http://peppergenome.snu.ac.kr) in

USDA-early, whereas it could be amplified in USDA-late. This indicates a sequence rearrangement differentiating the two alleles, and possibly a large insertion that prevents the amplification of this region in USDA-early. QTL mapping studies in *Arabidopsis* have enabled the identification of several examples of *cis*-regulatory polymorphisms in flowering genes such as *SVP*, *FLC* and *CONSTANS* that underlie flowering QTLs (Michaels et al. 2003; Mendez-Vigo et al. 2013; Rosas et al. 2014).

Despite the identification of numerous genes controlling flowering time in *Arabidopsis*, QTL studies for flowering time variation in natural populations have revealed few regions with large effects (Salomé et al. 2011). None of these regions include the *AP2*-related suppressor genes detected by mutant analyses. It remains to be determined whether the major effect in the *CaAP2* region on flowering time in pepper is specific to the population studied here or has a broad effect in a larger gene pool of *Capsicum*, by analyzing additional biparental populations or by genome-wide association studies.

Author contribution statement IP designed the research and wrote the manuscript. YB, VKS and HV conducted the experiments and analyzed the data. All authors read and approved the manuscript.

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Conflict of interest The authors declare no conflicts of interest.

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