The study of the E-class \textit{SEPALLATA3}-like MADS-box genes in wild-type and mutant flowers of cultivated saffron crocus (\textit{Crocus sativus} L.) and its putative progenitors

Athanasios Tsaftaris\textsuperscript{a,b,}\textsuperscript{*}, Konstantinos Pasentsis\textsuperscript{a}, Antonios Makris\textsuperscript{a}, Nikos Darzentas\textsuperscript{a}, Alexios Polidoros\textsuperscript{a,1}, Apostolos Kalivas\textsuperscript{a,2}, Anagnostis Argiriou\textsuperscript{a}

\textsuperscript{a} Institute of Agrobiotechnology, Center for Research and Technology Hellas, 6th Km Charilaou Thermi Road, Thermi GR-570 01, Greece
\textsuperscript{b} Department of Genetics and Plant Breeding, Aristotle University of Thessaloniki, Thessaloniki GR-541 24, Greece

\textsuperscript{*} Corresponding author at: Institute of Agrobiotechnology, Center for Research and Technology Hellas, 6th Km Charilaou Thermi Road, Thermi GR-570 01, Greece. Tel.: +30 2310 498271; fax: +30 2310 498270.
E-mail address: tsaf@certh.gr (A. Tsaftaris).
\textsuperscript{1} Present address: Department of Genetics and Plant Breeding, Aristotle University of Thessaloniki, Thessaloniki GR-541 24, Greece.
\textsuperscript{2} Present address: Cotton and Industrial Plants Institute, National Agricultural Research Foundation, Sindos GR-574 00, Greece.

\textbf{ABSTRACT}

To further understand flowering and flower organ formation in the monocot crop saffron crocus (\textit{Crocus sativus} L.), we cloned four MIKC\textsuperscript{c} type II MADS-box cDNA sequences of the \textit{E-class SEPALLATA3} (SEP3) subfamily designated \textit{CsatSEP3a/b,c/c}. as well as the three respective genomic sequences. Sequence analysis showed that cDNA sequences of \textit{CsatSEP3} c and c\textsubscript{as} are the products of alternative splicing of the \textit{CsatSEP3c} gene. Bioinformatics analysis with putative orthologous sequences from various plant species suggested that all four cDNA sequences encode for SEP3-like proteins with characteristic motifs and amino acids, and highlighted intriguing sequence features. Phylogenetically, the isolated sequences were closest to the SEP3-like genes from monocots such as \textit{Asparagus virgatus}, \textit{Orzya sativa}, \textit{Zea mays}, and the dicot \textit{Arabidopsis} SEP3 gene. All four isolated \textit{C. sativus} sequences were strongly expressed in flowers and in all flower organs: whorl1 tepals, whorl2 tepals, stamens and carpels, but not in leaves. Expression of \textit{CsatSEP3a/b,c/c} cDNAs was compared in wild-type and mutant flowers. Expression of the isolated \textit{CsatSEP3}-like genes in whorl1 tepals together with \textit{E-class CsatAPI/FUL} subfamily and \textit{B-class CsatAP3} and \textit{CsatPI} subfamilies of genes, fits the ABC\textsuperscript{e} “quartet model,” an extended form of the original ABC model proposed to explain the homeotic transformation of whorl1 sepals into whorl1 tepals in \textit{Lilium} and \textit{Asparagus} plants such as \textit{C. sativus}. This conclusion was also supported by the interaction of the \textit{CsatSEP3b} protein with \textit{CsatAPI/FUL} and \textit{CsatAP3} proteins. In contrast, expression of both B-class \textit{CsatAP3} and \textit{CsatPI} genes and the C-class \textit{CsatAGAMOUS} genes together with E-class \textit{CsatSEP3}-like genes in carpels, without any phenotypic effects on carpels, raises questions about the role of these gene classes in carpel formation in this non-grass monocot and requires further experimentation. Finally, taking advantage of the size and sequence differences in amplified genomic sequences of the triploid \textit{C. sativus} and comparing them with the respective sequences from \textit{C. romansi}, \textit{C. hadraticus} and \textit{C. cartwrightianus}, three putative wild-type diploid progenitor species, we examined the origin of \textit{CsatSEP3a} sequence.

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\begin{abstract}

Most angiosperm flowers contain four distinct organ types, from the outside to the inside, namely: sepals in whorl1, petals in whorl2, stamens in whorl3, and carpels in whorl4. Depending upon the taxa, this model can be adapted, placing species-specific homologous organs in each whorl. For example, plants such as orchids, roses, asparagus, maize, crocus (among many others) have varied and distinctive flowers. A common departure from the typical flower, observed in several monocots including saffron crocus (\textit{Crocus sativus} L.), is that there is no clear distinction between sepals and petals, and the petaloid organs in whorls 1 and 2 are therefore referred to as tepals (\textit{Bowman}, 1997). \textit{C. sativus} is a monocot triploid sterile species belonging to the \textit{Iridaceae} family of \textit{Asparagales}, whose whorl4 red stigmatic styles constitute saffron, a commercially very important popular food additive with delicate aroma and attractive color, also used for coloring and medical purposes. The flower of saffron is bisexual. The perianth consists of six petaloid tepals in whorls 1 and 2. The
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expressed in crocus leaves, too (Tsaftaris et al., 2004). CsatAP1/FUL genes were the only MADS-box genes to be expressed in the leaves from all the MADS-box genes examined till now in this species.

Here, extending our studies with crocus MADS-box genes, we report the isolation and characterization of SEP3-like genes, the second subfamily of the E-superclass MADS-box genes designated CsatSEP3-like genes. Four SEP3-like cDNAs corresponding to three isolated genomic sequences were isolated, characterized and their expression was studied. We also detected interaction between CsatSEP3-like protein described here and two other MADS-box proteins, CsatAP1/FRU (Tsaftaris et al., 2004) and CsatAP3 (Tsaftaris et al., 2006) that could explain the formation of tetramers between CsatSEP3 and CsatAP1/FRU in whorl1 and whorl2 tepals. Finally, the expression of all four CsatSEP3 sequences was compared in wild-type and a double flower, as well as in a stamenless flower mutant. Taking advantage of size and sequence differences in isolated genomic sequences of CsatSEP3a, we examined the presence of each genomic region in the three putative diploid progenitors of C. sativus, namely: C. cartwrightianus, C. tomasii and C. hadriaticus, to obtain more evidence for the origin of the three genomes in the cultivated triploid crocus, C. sativus (Grilli Caiola et al., 2004).

Materials and methods

Plant material

Crocus sativus field-growing plants were collected from Kozani, Greece. Tissues from wild-type saffron and two mutant plants found in the field, one lacking stamens and one with double the number of flower organs (tepals, stamens, carpels) were collected. Sampling took place during the late flowering season in October. Tissues were separated and immediately frozen in liquid nitrogen and stored at −80 °C until use. Tissues from C. cartwrightianus Herb., and C. hadriaticus Herb., subsp. hadriaticus were collected from the CROCUSBANK project database (http://www.crocusbank.org/). Genomic DNA from C. tomasii Ten. was kindly provided by Dr. Ole Seberg.

RNA isolation and cDNA synthesis

Total RNA from flowers and leaves were extracted using Trizol reagent (Invitrogen) following the manufacturer’s instructions. First-strand cDNA synthesis was performed with 200 U SuperScript II-RT (Invitrogen) in a 20 µl reaction volume containing a pool of 5 µg total RNA from flowers and leaves (2.5 µg each), 0.5 µg NdT-Adaptor primer (5′-TGCATGAATGTTGTGTAATG(T)18-3′) 5′ phosphate (synthesized at VBC-genomics, Austria), 0.5 mM dNTPs, 1 × first strand buffer (Invitrogen) and 10 mM DTT (Invitrogen). The reactions were incubated for 50 min at 42 °C followed by 15 min at 70 °C to inactivate the RT. After the addition of 2 units of RNAseH (Invitrogen), the reactions were incubated for 50 min at 42 °C and stored at −80 °C. First-strand cDNA synthesis was performed as has been previously described (Polidoros et al., 2006). Briefly, after cDNA synthesis, the reactions were purified using the QiAquick PCR purification kit (Qiagen). Rolling-circle amplification-RACE (RCA-RACE) of cDNAs

Circularization followed with rolling circle amplification-RACE (RCA-RACE) of C. sativus cDNA was performed as has been previously described (Polidoros et al., 2006). Briefly, after circularization, the reactions were purified using the QiAquick PCR purification kit (Qiagen). Rolling circle amplification reactions were performed in 50 µl volume containing 1/6 (5 µl) of the circularized cDNA, 1 mM dNTPs, 200 µg/ml BSA (NEB), 1 × phi29 DNA Polymerase reaction buffer (NEB), 10 U phi29 DNA Polymerase (NEB) and 1 µM NSFInvR primer (5′-TTCATATTGTCAG-3′), with two PTO linkages on the 3′-end (custom synthesized at VBC-genomics). The reactions were incubated for 1 h at 30 °C followed by the addition of 1 µM NSFInvR (5′-TGGTTAATGAT-3′) and further incubated for 1 h at 30 °C. Finally heat inactivation was performed for 10 min at 60 °C.

For the direct cloning approach with RCA-RACE using degenerate inward primers, where the forward primer extends versus the 3′ end and the reverse primer extends versus the 5′ end, a 1 µl aliquot of a 1/10 dilution of the rolling circle amplification reaction was used for the PCRs. The reactions were performed in 50 µl volume containing 0.2 mM dNTPs, 1 U DyNAzyme II DNA polymerase (Finnzymes), 0.4 µM MADS-2F degenerate primer (5′-GTCTTGYGTAYYCAGGT-3′) designed from the conserved amino acid sequence VLCDAEV in antisense orientation and either 0.4 µM MADSSR degenerate primer (5′-AAYGTNACATGCNGRTTDA-3′) designed from the conserved amino acid sequence INKVQTF in antisense orientation or 0.4 µM MADS6R degenerate primer (5′-CGRTTDATYRTYTC-3′) designed from the conserved amino acid sequence IENKINR in antisense orientation. The cycling conditions were: 3 min at 94 °C, 40 cycles of 45 s at 94 °C, 45 s at 50 °C, 1.5 min at 72 °C and a final extension step of 10 min at 72 °C.

For the second approach, degenerate outward primers were used. In this case, the Forward primer extends versus the 5′ end and Reverse primer extends versus the 3′ end. In order to isolate the family of SEPALATA like genes, one (1) µl of the RCA-RACE library from crocus, prepared with the InvVUP primer as described in a previous paper (Polidoros et al., 2006), was used as template for inverse PCR, under the same conditions as above. Specifically, we used primers MADS-2F and MADS PromR degenerate primer (5′-CTTVSACWAGTITSACYTG-3′) corresponding to the conserved amino acid sequence QVTFSK in antisense orientation. Several PCR products ranging from 900 bp to 1300 bp were excised from an agarose gel, ligated into pcR 2.1-TOPO vector using the PCR II-TOPO TA cloning kit (Invitrogen) and transformed in DH5α competent cells. Several individual clones were screened for the presence of an insert and sequenced. Finally based on the sequence information obtained from the isolated sequences, gene-specific primers were designed and used for PCR reactions to complete the small missing parts (39–57 nucleotides between the two outward-oriented primers) and obtain the full length coding sequence of the genes. Having as template 1/30 of the single stranded cDNA used in the RCA-RACE experiment, the reactions also contained 0.2 mM dNTPs, 1 U DyNAzyme II DNA polymerase (Finnzymes) and 0.4 µM of the corresponding primers. The cycling conditions were: 2 min at 94 °C, 35 cycles of 45 s at 94 °C, 30 s at 54 °C, 1.5 min at 72 °C and a final extension step of 10 min at 72 °C. The primers used were: Sep3-F (5′-TAAATTGCGTCTTACGAA-3′) and Sep3-2R (5′-ATCGAAGGCTGATAATTAACC-3′) for amplifying CsatSEP3a, Contig5F (5′-TCTTACGAGAGGAATATCAGT-3′) and Contig5R (5′-ATGTTCTTTGTTATCCAGGAC-3′), for amplifying CsatSEP3b and Contig5S/6F (5′-GAGAATGAGGAATATCAGT-3′) and Contig5S/6R (5′-GAGAATGAGGAATATCAGT-3′) for amplifying CsaSEP3c and CsaSEP3c as. A single PCR product from each primer pair was cloned as above. Several individual clones were screened for the presence of an insert and sequenced (Macrogen, Korea).

Isolation of genomic sequences

Genomic DNA was isolated from leaves using a modified CTAB-based protocol (Li et al., 2001). For amplification of the C. sativus SEP3 genes, specific primers were designed based on the information gained from the isolated cDNAs. PCR amplification was performed using 0.1 µg genomic DNA as template, 200 nM primers each, 1 mM dNTPs and 1 unit DyNAzyme II polymerase (Finnzymes, Espoo, Finland) in 50 µl total volume reaction. PCR conditions were 94 °C for 3 min, 35 cycles of 94 °C for 30 s, 58 °C for 30 s, 72 °C
for 2 min and a final extension period at 72 °C for 10 min. The PCR products were separated on a 1% agarose gel and cloned into the pCR 2.1-TOPO vector using the TOPO TA cloning Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s protocol.

Five individual clones for each CsatSEP3a, CsatSEP3b, CsatSEP3c and CsatSEP3c_as were sequenced. The same set of primers and PCR conditions, used to isolate the three genomic sequences corresponding to the last four exons and introns from C. sativus, were used to amplify genomic sequences of the SEP3 genes from genomic DNA isolated from C. cartwrightianus, C. tomasii, and C. hadriaticus subspp. hadiaticus. Genomic sequences for SEP3A like genes were cloned as described and three individual clones were sequenced from each crocus species. Sequencing was performed by MACROGEN (Korea) on an ABI3730 DNA analyzer.

Bioinformatics analysis

We collected all Viridiplantae proteins from UniProt version 15.4 of 2009 with a statistically significant hit for the MADS-box domain (PF00319: SRF-type transcription factor (DNA-binding and dimerisation domain)) from the Pfam database (Finn et al., 2008). After sequence fragments were filtered out, the resulting set was the subject of an all-against-all similarity detection step using the BLAST algorithm (Altschul et al., 1997). We used the bidirectional best hit approach (Overbeek et al., 1999) to discover putative orthologous proteins. We also asked for any orthologous sequence to be one of at least ten sequences with the MADS-box domain from the corresponding monocot or dicot species, so that we reduced the chance of best hits due to lack of sequence context. We thus concatenated our C. sativus sequences and their putative orthologs, and the four SEP proteins of Arabidopsis and their putative orthologs.

The multiple alignment of the sequence set described above was created with version v6.713b (2009/09/24) of the MAFFT algorithm [http://www.ebi.ac.uk/Tools/mafft/multalin] and the G-INS-I global alignment strategy with a maximum of 1000 iterations, edited and visualized with JalView version 2.6 (Waterhouse et al., 2009). The phylogenetic tree was constructed with the Geneious (trial/free version 5.3.3 – www.geneious.com) Tree Builder with the Neighbor Joining method, the default Jukes-Cantor genetic distance model, and bootstrap resampling with 1000 replicates and 50% support threshold. The resulting tree was edited and visualized with FigTree version 1.3.1 (http://tree.bio.ed.ac.uk/software/figtree/).

The multi-RELIEF algorithm (Ye et al., 2008) was used on the resulting alignment, with 100 iterations and sample size of 10, and useful alignment positions with scores equal or greater than 0.8 (arbitrary threshold based on manual observations) were selected.

Expression analysis

The expression analysis of the isolated genes was performed with RT-PCR. Total RNA from leaves, flowers, whorl1 tepals, whorl2 tepals, stamens, and carpels of a mutant flower lacking stamens and a double flower, were extracted using the RNeasy plant mini kit.
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Additionally subcloned into the pYES-mycGST vector expressing a myc tag-GST fusion protein in yeast. The “bait” and “library” two hybrid constructs were co-transformed into the yeast reporter strain EC148 (ura3 trp1 his3 6LexA-operator-LEU2) by lithium acetate transformation. Transforms were selected in glucose complete media lacking histidine and tryptophan (CM-his, trp). Assays for the suitability of “bait” to test interactions were performed on galactose-raffinose complete media lacking leucine (CM-leu) (Kilić et al., 2004). The library (pJG4-5) constructs were co-transformed with the pYESmGSTCROSA, B3FTV5 and CROSA, respectively). It is worth noting that part of the 3′ end of CsatSEP3c was identified in the recently published partial collection of saffron stigma-specific 6,803 ESTs (D’Agostino et al., 2007) under the cluster ID cr.saCl001179. The other three sequences were not represented in that partial collection.

To assess whether the four isolated SEP3-like cDNAs correspond to four different genomic sequences, primers were used to isolate part of their genomic sequences covering the regions where differences among the cDNA sequences were found. Three different genomic sequences were isolated, corresponding to CsatSEP3a, CsatSEP3b and CsatSEP3c containing the last four exons and introns of the genes (Fig. 1A). Alignment between the four isolated cDNAs and the three genomic sequences revealed that the same genomic sequence encodes for the two CsatSEP3a and CsatSEP3c sequences differing in 15 bp as a result of an alternative splicing, thus, “as” indicates alternative splicing (Fig. 1B). As also shown in Fig. 1A, similar genomic organization was found for all the isolated genes with two (CsatSEP3a and CsatSEP3b) presenting higher similarity, whereas CsatSEP3c was more distant.

**Amino acid sequence and phylogenetic analysis**

We collected 1895 sequences from the UniProt protein database containing the MADS-box domain, as described in the Materials and Methods. The Arabidopsis SEP3 protein (UniProt accession number: O22456) was the top Arabidopsis hit for all four cros proteins with 69.26% identity to CsatSEP3a, 67.74% to CsatSEP3b, 67.35% to CsatSEP3c, and 65.31% to CsatSEP3c as; the other three Arabidopsis SEPALLATA3 proteins SEP1 (UniProt: Q5XXN7), SEP2 (UniProt: P29384), and SEP4 (UniProt: P29383) were consistently in the hit list, with SEP4 furthest away.

Using BLAST-derived orthology information, we aligned our four sequences with Arabidopsis SEP1–4, along with putative orthologs from different monocot and dicot plant species (Sup. 1). As shown in Fig. 2, the resulting alignment highlighted interesting features, including the near-perfect conservation of the MADS-box domain (alignment positions 9–60), along with high conservation of the three subdomains of the K-box as described in (Yang and Jack, 2004) (K1: 99–119; K2: 132–146; K3: 154–194). Also very highly conserved are two hydrophobic leucines (alignment positions 124 and 261) in the K1–K2 interhelical region (Yang and Jack, 2004).

The SEP3-specific interacting arginine (R) at position 150 (Yang and Jack, 2004) was found in all C. sativus SEP3-like sequences as well as their putative orthologs, but was replaced by a lysine in the majority of the Arabidopsis SEP1, SEP2, and SEP4 and their putative orthologs. Other potential specificity-determining residues, as recognized by the multi-RELIEF algorithm, existed at position 80 with a proline in all SEP3 orthologs compared to isoleucines, leucines, methionines, valines and a glutamic acid in the rest, and at position 298 where the SEP3 orthologs had a leucine in the great majority of cases, whereas the rest exclusively featured a (biochemically similar) methionine. It should be noted that the significance of these residues in these positions cannot be evaluated, but are mentioned to support the placement of the C. sativus sequences in the SEP3 group (since they share the same residues in these positions with the rest of the SEP3 orthologs – an interesting exception is the phenylalanine at position 298 in CsatSEP3c and CsatSEP3c as), and as potentially interesting features for further research.

As also shown in Fig. 2, the phylogenetic tree of putative orthologs clearly places two Asparagus proteins (AOM1 in Asparagus...
officinalis and MADS1 in Asparagus virgatus) as the closest putative ortholog to all four predicted C. sativus SEP3-like protein sequences.

**Characterization of the genomic sequences of C. sativus CsatSEP3a and its putative progenitors**

Amplification of the CsatSEP3a genomic region from C. sativus using gene specific primers showed two bands of different size designated CsatSEP3a1 and CsatSEP3a2 (Fig. 3A), while only a single band was observed for CsatSEP3b and CsatSEP3c (data not shown). To identify the origin of the CsatSEP3a1 and CsatSEP3a2 isolated sequences, we attempted to amplify SEP3a like genomic sequences from three putative progenitors of C. sativus namely C. cartwrightianus, C. tomasii and C. hadriaticus (Seberg and Petersen, 2009; Grilli Caiola et al., 2010). As shown in Fig. 3, based on size differences, it is likely that the CsatSEP3a1 gene derived from C. hadriaticus and CsatSEP3a2 from C. tomasii. To obtain evidence for the origin of CsatSEP3a1 and CsatSEP3a2, the two amplified bands from C. sativus and those from C. cartwrightianus, C. tomasii and C. hadriaticus were cloned and sequenced. Alignment of the two CsatSEP3a genomic sequences from C. sativus and the three putative progenitors revealed higher similarity with C. tomasii and C. hadriaticus as expected. Interestingly, only one CsatSEP3a cDNA was isolated from C. sativus (named CsatSEP3a1 Acc. EU424137) identical in the coding region of the CsatSEP3a1 genomic sequence and more similar to the genomic sequence isolated from C. hadriaticus. This observation suggests that only the CsatSEP3a1 gene, originated from C. hadriaticus, is expressed in C. sativus. Although these are preliminary data, they indicate that it is likely that one of the C. sativus triploid progenitors is C. hadriaticus, while C. tomasii could be another, as shown in the phylogenetic tree in Fig. 3B.

**Expression analysis**

The expression patterns of the isolated SEP3-like genes were compared between different crocus organs by RT-PCR. As shown in Fig. 4, the transcripts were mainly present in flowers and not in leaves (although a weak amplification product for CsatSEP3b and CsatSEP3c was observed in leaves). We also investigated expression patterns in different flower organs. As shown in Fig. 4, RT-PCR experiments performed with cDNA synthesized from tepals, stamens and carpels resulted in the identification of all four transcripts in all mature flower organs.

Due to its triploidy, cultivated C. sativus is a sterile perennial species asexually propagated for thousands of years following its domestication, and numerous mutants preserved in the fields have been identified and described (Grilli Caiola et al., 2004). Among them, indeterminate flower phenotypes with double (Fig. 5) or even triple flowers have been found. Since the SEP gene has been found to play a role in flowering and flower determinacy (Liu et al., 2009) in addition to its role in flower organ formation according to the ABCE model (Malcomber and Kellogg, 2004), the expression analysis of all four CsatSEP3-like transcripts was examined in one such mutant with double flowers. As shown in Fig. 5, there were no expression differences among the four SEP-like transcripts in WT and the double flower (DF) mutant. Also, no differences were found when another mutant (M), lacking the stamen part of the C. sativus flower, was examined (Fig. 5), where according to the ABCE model, SEP like proteins are expected to form tetrameric complexes together with B-class CsatAP3/PI-like and C-class CsatAG-like proteins.

**Evaluation of protein–protein interactions**

To assess the capacity of the isolated CsatSEP3-like proteins to interact with other MADS-box proteins isolated from crocus, and therefore add further evidence to their characterization, we selected CsatSEP3b along with previously published CsatAP3b and CsatAP1/FULc to test on the “yeast two-hybrid” system and on a cell-based GST-fusion co-precipitation approach. The three proteins were expressed as fusions to the LexA protein and the B42AD- HA (hemaglutinin tag) and were transformed singly and in pairs in yeast reporter cells (Golemis et al., 2001). Unfortunately, the LexA fusions of the MADS-box proteins exhibited a capacity to autoactivate the LEU2 reporter, making the two-hybrid assay unusable (data not shown). Thus, we subsequently employed a cell-based direct co-precipitation assay based on GST fusions. The cDNA of CsatAP1/FULc was expressed as the C-terminal fusion to a myc epitope tag and to the GST protein under the control of the inducible galactose promoter (Fig. 6A). The B42AD-HA fusions of CsatSEP3b, CsatAP3b and CsatAP1/FULc and OYE2 (yeast Old Yellow Enzyme 2, used as control (Odat et al., 2007) were stably co-expressed with
either the empty GST or the CsatAP1/FULc fusion. Yeast offers the advantage in co-precipitation assays that plasmids are maintained stably and proteins are consistently expressed in modest quantities compared to bacterially expressed GST fusions. Interactions take place within the intracellular environment of a eukaryotic cell.

Protein extracts from cells expressing the various combinations were incubated with glutathione (GSH) agarose beads, which bind strongly to the GST component. Proteins that specifically bind to the CsatAP1/FULc part of the GST fusion would co-precipitate and could be detected using an HA antibody recognizing their N-terminal fusion. The B42AD-HA fusion of the yeast protein OYE2 was used as negative control, whereas the well-known interaction between a GST-OYE2 and B42AD-HA-actin was used as positive control for the assay.

As shown in Fig. 6B, GST-CsatAP1/FULc specifically co-precipitated CsatSEP3b and CsatAP3b but did not appear to interact significantly with itself to form a homodimer. As expected, GST alone could not precipitate the two proteins and GST-CsatAP1/FULc did not co-precipitate the unrelated OYE2 protein, confirming overall the specificity of the interactions observed.

**Discussion**

SEP-like genes play an important role in flower development, encoding one member of the transcriptional regulatory complex controlling flower organ formation (Immink et al., 2009).

In this work, we attempted to isolate SEP-like genes from C. sativus. Despite our extensive efforts, the only SEP genes isolated from crocus flowers were SEP3-like, as bioinformatics analysis with putatively orthologous sequences strongly suggests. Indeed, as shown Fig. 2, outside the promiscuous MADS-box domain, there are conserved amino acids (e.g. the SEP3-specific arginine (R) at position 147 of the alignment), but also intriguing differentiation. Such sequence deviations are primarily found in CsatSEP3c and CsatSEP3c-as (see Results), which could indicate some form of sub-functionalization, albeit not evident in our expression analysis in different flower organs and mutant flowers. These differences in the sequences can also be explained by the triploid genome of saf-
from crocus. *C. sativus* is triploid (2n = 3x = 24), generally assumed to be of autotriploid or hybrid origin (Grilli Caiola et al., 2004, 2010; Frizzi et al., 2007). Most researchers assume that one of the *C. sativus* parents must have been *C. cartwrightianus* donating the 2n = 2x = 16 chromosomes part of the genome, while another unknown diploid parent contributed the other 1n = 8 = 8 chromosomes. But recently, the hypothesis, based on sequence similarities, that *C. sativus* is an allotriploid consisting of three different genomes, two perhaps of close similarity and a third one more distant, is becoming credible (Grilli Caiola, 2005; Grilli Caiola et al., 2010). For instance, in previous work from our group (Tsafaritis et al., 2004, 2006; Kalivas et al., 2007) with CsatAP1/FUL, CsatAP3, CsatPI and CsatAG-like genes, three forms were frequently isolated. Two show very close similarity, presumably originating from *C. cartwrightianus* or *C. tomasii*, progenitors contributing maybe the two more similar genomes and one more distantly related, presumably originated from the other progenitor species, reinforcing the idea that *C. sativus* is an allotriploid species and explaining the presence of differences in the sequences of the isolated SEP3-like genes. Experiments are underway in our and other laboratories to clarify the progenitor species of the triploid cultivated *C. sativus*, and complement the preliminary results presented in this paper, to better understand the origin of the different classes of MADS-box genes in this crop and the origin of this crop itself.

Unlike the sequence and probably the functional conservation, the expression pattern of SEP-like transcripts differs between monocots and eudicots. Within monocots, SEP-like genes have been most intensively studied in grasses, including the important cereals maize and rice (Cui et al., 2010; Gao et al., 2010). Similarly to other monocots, expression of the isolated SEP3-like genes of *C. sativus* was detected in all four whorls of flower organs. This pattern of extended expression of E-class genes together with B-class genes reported previously (Tsafaritis et al., 2004, 2006) is compatible with tepal formation in whorl1. *Muscaria armeniacum* is another member of *Asparagales* that has petaloid organs in the outer two whorls and the expression of B-class genes extended to whorl1, similar to saffron crocus (Nakada et al., 2006). Thus, the extended expression of B- and E-class genes in whorl1 fits the extended ABCE model proposed to explain tepal formation in tulip and other non-grass monocots such as *C. sativus* (van Tunen et al., 1993; Kanno et al., 2003, 2007). This is further supported by the co-precipitation of CsatSEP3b and CsatAP3b proteins to form heterodimers with CsatAP1/FUL. This result is supported indirectly by our earlier findings concerning the expression of a *C. sativus* NAP-like gene, a member of the plant specific NAC family transcription factor proteins (Kalivas et al., 2010). It is known, for example, that heterodimers of AP3/PI MADS-box transcriptional factors induce the expression of NAP-like genes by recognizing and binding on the CArG motifs present on the NAP promoter sequences (Sablowski and Meyerowitz, 1998). In the promoter sequence of CsatNAP-like, isolated and characterized previously from our group, two nearby putative CArG boxes were found, presumably targets of AP3/PI heterodimers that can induce the expression on CsatNAP-like gene in whorl1, where CsatNAP was found to be expressed, indicating indirectly that a functional AP3/PI protein could be present in whorl1 tepals in *C. sativus*, too. Combining this observation with the presence of CsatSEP3 transcripts in the whorl1 and the protein–protein interaction experiments of CsatSEP3b with CsatAP3 and CsatAP1/FULc presented in this paper (Fig. 6), we suggest that, in whorl1 of saffron crocus, the formation of the quartet protein complex between SEP3, AP3/PI and AP1/FUL is feasible. However, expression of genes like CsatAP3/PI, CsatAP1/FUL and CsatSEP3 in whorl1 (as observed in *C. sativus* wild-type and mutant flowers and other non-grass monocots), together with CsatAG-like MADS-box gene expression, is a result difficult to explain (Kanno et al., 2007). To further our understanding of saffron crocus flower formation and particularly whorl1 carpel formation, it would therefore be interesting to examine MADS-box protein accumulation and interaction of crocus B-, C- and E-class function genes in whorl1.

In summary, four *C. sativus* SEP3-like cDNAs, transcribed from three genes, were isolated, their sequences and phylogenetic relationships described, and their expression patterns and potential for protein interactions with other *C. sativus* MADS-box proteins characterized. All isolated cDNAs encode for proteins belonging to the mixed eudicot–monocot SEP3 subclade of MADS-box proteins and although there seems to be no reason for the absence of other crocus SEP-like genes, these were the only such genes isolated after extensive search with different methods. Overall, these data suggest that the inferred role of the isolated E-class genes in *C. sativus* is compatible with that of E-function MADS-box transcription factors.

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**Appendix A. Supplementary data**

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jplph.2011.03.015.

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