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Heterotopic expression of class B floral homeotic genes supports a modified ABC model for tulip (*Tulipa gesneriana*)

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Abstract

In higher eudicotyledonous angiosperms the floral organs are typically arranged in four different whorls, containing sepals, petals, stamens and carpels. According to the ABC model, the identity of these organs is specified by floral homeotic genes of class A, A+B, B+C and C, respectively. In contrast to the sepal and petal whorls of eudicots, the perianths of many plants from the Liliaceae family have two outer whorls of almost identical petaloid organs, called tepals. To explain the Liliaceae flower morphology, van Tunen *et al.* (1993) proposed a modified ABC model, exemplified with tulip. According to this model, class B genes are not only expressed in whorls 2 and 3, but also in whorl 1. Thus the organs of both whorls 1 and 2 express class A plus class B genes and, therefore, get the same petaloid identity. To test this modified ABC model we have cloned and characterized putative class B genes from tulip. Two *DEF*- and one *GLO*-like gene were identified, named *TGDEFA*, *TGDEFB* and *TGGLO*. Northern hybridization analysis showed that all of these genes are expressed in whorls 1, 2 and 3 (outer and inner tepals and stamens), thus corroborating the modified ABC model. In addition, these experiments demonstrated that *TGGLO* is also weakly expressed in carpels, leaves, stems and bracts. Gel retardation assays revealed that TGGLO alone binds to DNA as a homodimer. In contrast, TGDEFA and TGDEFB cannot homodimerize, but make heterodimers with PI. Homodimerization of GLO-like protein has also been reported for lily, suggesting that this phenomenon is conserved within Liliaceae plants or even monocot species.

Introduction

In higher eudicotyledonous flowering plants the floral organs are arranged in four different whorls, containing sepals, petals, stamens and carpels, respectively. The specification of floral organ identity is explained by the ABC model (Figure 1; Coen and Meyerowitz 1991; Weigel and Meyerowitz 1994). Expression of class A genes specifies sepal formation in whorl 1. The combination of class A and B genes specifies the formation of petals in whorl 2. Class B and C genes specify stamen formation in whorl 3, and expression of the class C gene alone determines the formation of carpels in whorl 4. Class A, B, and C genes have been isolated from several eudicotyledonous model plants such as *Arabidopsis*, *Antirrhinum* and *Petunia*, and most of them belong to the family of MADS-box genes encoding transcription factors (for a review, see Theissen *et al.*, 2000). All class B genes known to date belong to either the *DEFICIENS*- (*DEF*-) or *GLOBOSA*- (*GLO*-)like genes, two closely related clades of MADS-box genes. The corresponding (orthologous) genes in *Arabidopsis* are *APETALA3* (*AP3*)

The nucleotide sequence data of the cDNAs reported in this paper have been deposited in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession numbers AB094965 (*TGDEFA*), AB094966 (*TGDEFB*), and AB094967 (*TGGLO*).

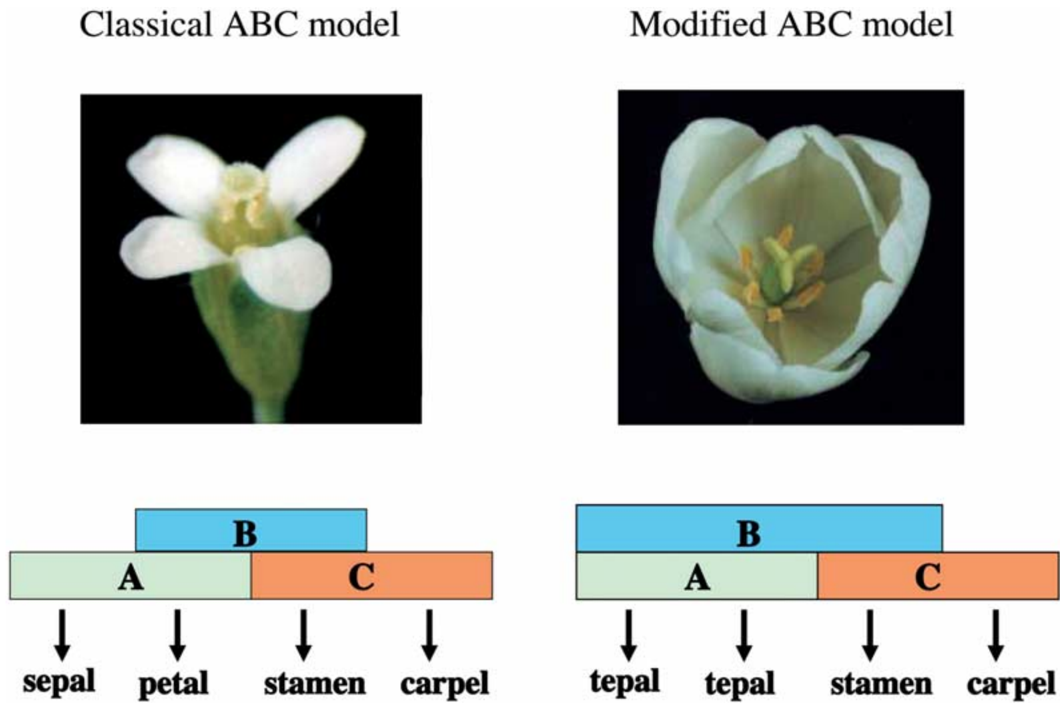

Figure 1. Classical and modified ABC model. In higher eudicotyledonous flowering plants (left, Classical ABC model, *Arabidopsis thaliana*), expression of an A-function gene specifies sepal formation in whorl 1. The combination of A- and B-function genes specifies the formation of petals in whorl 2, B- and C-function genes specify stamen formation in whorl 3, and expression of a C-function alone determines the formation of carpels in whorl 4. In contrast, Liliaceae plants (right, Modified ABC model, *Tulipa gesneriana*) have two whorls of almost identical petaloid organs, called tepals. A modified ABC model has been proposed by van Tunen *et al*. (1993) to explain the flower morphology of tulip. In this model, class B genes are expressed in whorl 1 as well as whorl 2 and 3, thus the organs of whorl 1 and whorl 2 have the same petaloid structure.

and *PISTILLATA* (*PI*), respectively (for a review, see Theissen *et al*., 1996, 2000).

In contrast to the flowers of higher eudicots, the perianths of many Liliaceae have two whorls of almost identical petaloid organs, called tepals. Many monocot flowers have three outer tepals, three inner tepals, 3 + 3 stamens, and three carpels (Dahlgren *et al*., 1985). To explain the morphology of wild-type and some mutant flowers of tulip (*Tulipa gesneriana*, Liliaceae), van Tunen *et al*. (1993) proposed a modified ABC model (Figure 1). In this model, class B genes are expressed in whorl 1 as well as in whorls 2 and 3, so that the organs of whorls 1 and 2 have the same petaloid structure. Although the modified ABC model may apply to lily (*Lilium*) and other Liliaceae as well, there are so far no molecular data to support it.

Concerning monocotyledonous plants, several class B genes were isolated from diverse cereal grass species up to now, such as rice (*Oryza sativa*) and maize (*Zea mays* ssp. *mays*). However, very few data are available for other monocots.

The tiny, wind-pollinated flowers of the grasses (Poaceae) are quite different from the flowers within other flowering plant families. They have carpels and stamens like their eudicot relatives, but they lack typical petals and sepals. Instead, lodicules, palea and lemma surround the carpels and stamens, thus constituting structures called florets. Lodicules are small, glandular-like organs that swell at anthesis to spread the lemma and palea apart so that the wind can disperse the pollen produced by the stamens.

For *OSMADS2*, a *GLO*-like gene from rice, mRNA accumulation was observed in lodicule and stamen primodia (Kyozuka *et al*., 2000). This RNA accumulation pattern persisted until late stages of flower development. The transcripts of *OSMADS4*, another *GLO*-like gene from rice, were observed in lodicules, stamens and carpels (Moon *et al*., 1999). Transgenic plants expressing antisense RNA of *OSMADS4* exhibited homeotic transformations of lodicules into palea/lemma-like structures and stamens into carpel-like organs (Kang *et al*. 1998). *OSMADS16*, the *DEF*-like gene from rice, was isolated from a young

inflorescence cDNA expression library by the yeast two-hybrid screening method using *OSMADS4* as a bait (Moon *et al.*, 1999). RNA blot analysis showed that the *OSMADS16* gene is expressed in lodicules and stamens (Moon *et al.*, 1999). Ectopic expression of *OSMADS16* caused alteration of carpels to stamen-like organs (Lee *et al.*, 2000). *Silky1* was identified as the *DEF*-like gene from maize (Ambrose *et al.*, 2000). The florets of *silky1* mutant plants show homeotic conversions of stamens into carpels, and lodicules into palea/lemma-like structures, transformations very similar to those of *OSMADS4* antisense plants. *ZMM16*, *ZMM18* and *ZMM29* are *GLO*-like genes from maize. They are expressed in lodicules, stamens and carpels throughout spikelet development in male and female inflorescences (Münster *et al.*, 2001). The phenotypes of the mutant and transgenic plants and the expression patterns support the view that the *DEF*- and *GLO*-like genes from rice and maize have very similar functions as the floral homeotic B-function genes in eudicots. The phenotype data and expression patterns nicely fit into an ABC model in which the lodicules are considered as petal homologues (Baum, 1999; Ambrose *et al.*, 2000). Alternatively, during evolution lodicules may have been derived from stamens (Baum and Whitlock, 1999; Kramer and Irish, 2000). Anyway, grass plants have highly derived flowers without well-developed perianths and thus do not directly bear on understanding the characteristic differences between Liliaceae and eudicot perianth morphology in terms of floral homeotic genes and the ABC model.

Recently, however, *LMADS1*, a *DEF*-like gene, was isolated from a lily species, *Lilium longiflorum* (Tzeng and Yang, 2001). By northern blot analysis strong expression of *LMADS1* was detected in whorl 2 (inner tepals) and whorl 3 (stamens). Surprisingly, however, *LMADS1* mRNA expression was found to be relatively weak not only in whorl 4 (carpels), but also in whorl 1 (outer tepals). Accordingly, by western blot analysis the LMADS1protein was detected only in whorls 2 and 3, but not in the organs of whorl 1. This is remarkable, given that the morphology of the floral organs in whorl 1 and whorl 2 is petaloid and nearly identical in lily. Based on their results, Tzeng and Yang (2001) suggested that the regulation of outer and inner tepal development is considerably different in lily despite the high similarity of the respective organs.

Unfortunately, the technique of genomic Southern is difficult to apply to lily because of the huge genome size. This may be one reason why it is unclear whether other *DEF*-like genes exist in lily. Also no information about the expression of *GLO*-like genes, the other type of class B genes, has been published for lily. Since their gene products can bind to DNA as homodimers, while heterodimers of DEF- and GLO-like proteins are absolutely required for the B-function in eudicots (Winter *et al.*, 2002), also a B-function based solely on *GLO*-like genes would theoretically be conceivable in lily outer tepals. As a consequence, the applicability of the ABC model to lily remains an open question.

To gain further insight into the floral homeotic genes of Liliaceae plants we have cloned and characterized the orthologues of eudicot class B genes, i.e. *DEF*- and *GLO*-like genes, from tulip, *Tulipa gesneriana*. Our findings support the view that the modified ABC model (van Tunen *et al.*, 1993) explains tulip (and perhaps also lily) flower morphology.

Materials and methods

Plant material

Plants of *Tulipa gesneriana* cv. White Dream were used. Plants were grown in the greenhouse facilities of MPIZ in Cologne, Germany. For Southern and northern blot analyses, floral organs (carpels, stamens, inner and outer tepals), leaves, bracts, stems, bulbs and roots were frozen in liquid nitrogen immediately after collection and stored at −80 °C. For northern blot analysis, we used 20 mm long flower buds, which represent a late developmental stage.

Cloning of cDNAs

Partial cDNAs were isolated by the 3′ rapid amplification of cDNA ends (RACE) method (Frohman *et al.*, 1988; Münster *et al.*, 1997). As template, poly(A)$^+$ RNA prepared from 4 cm flower buds of *Tulipa gesneriana* was used. Upstream sequences overlapping with the 3′ fragments were isolated by 5′-RACE with the 5′/3′-RACE kit (Roche Diagnostics, USA). cDNA clones with complete open reading frames (ORFs) were isolated by PCR with primers located in the 5′- and 3′-UTR regions and with cDNA pools as template. Sequences of primers used during the RACE procedures can be downloaded from the home page http://www.uni-jena.de/biologie/genetik/primerlist.htm. For each gene, at least three independent cDNAs were cloned, and both strands were sequenced by the MPIZ DNA core

facility (ADIS) on PE Biosystems ABI Prism 377 and 3700 sequencers by BigDye-terminator chemistry.

Phylogenetic analysis

Predicted amino acid sequences were used for phylogenetic analysis. Protein sequences were aligned by Clustal W, and phylogenetic trees were constructed by the neighbor-joining method. Bootstrap values were derived from 1000 replicate runs (Thompson *et al.*, 1994; http://www.ddbj.nig.ac.jp/E-mail/homology.html). All neighbor-joining trees were drawn by NJplot (Perrière and Gouy, 1996). The GenBank accession numbers of the amino acid sequences used are: *AG* (X53579), *AP1* (Z16421), *AP3* (M86357), *BOBAP3* (U67456), *BOI1AP3* (U67453), *BOI2AP3* (U67455), *CMB2* (L40405), *CUM26* (AF043255), *DAL11* (AF158539), *DAL12* (AF158541), *DEF* (X52023), *FBP1* (M91190), *GGLO1* (AJ009726), *GGM15* (AJ251555), *GGM2* (AJ132208), *GLO* (X68831), *LMADS1* (AF503913), *LRDEF* (AB071378), *LRGLOA* (AB071379), *LRGLOB* (AB071380), *NMH7* (L41727), *NTGLO* (X67959), *OSMADS16* (AF077760), *OSMADS2* (L37526), *OSMADS3* (L37528), *OSMADS4* (L37527), *PCAP3* (AF052872), *PI* (D30807), *PLE* (S53900), *PMADS1* (X69946), *PMADS2* (X69947), *PNAP3-1* (AF052873), *PNAP3-2* (AF052874), *PNPI1* (AF052855), *PNPI2* (AF052856), *PTAP3-1* (AF052870), *PTAP3-2* (AF052871), *RAD1* (X89113), *RAD2* (X89108), *RBPI1* (AF052859), *RBPI2* (AF052860), *SILKY1* (AF181479), *SLM2* (X80489), *SLM3* (X80490), *SQUA* (X63701), *STDEF* (X67511), *TGDEFA* (AB094965), *TGDEFB* (AB094966), *TGGLO* (AB094967), *TM6* (AF230704), *WAP3* (*TAMADS51*, AB007506), *ZAG1* (L18924), *ZAP1* (L46400), *ZMM16* (AJ292959), *ZMM18* (AJ292960), and *ZMM29* (AJ292961).

Southern blot analysis

Total DNA was isolated from leaves by the method of Murray and Thompson (1980). Genomic DNA (50 μg) was digested with one of four restriction enzymes (*Eco*RI, *Xba*I, *Bam*HI, *Hin*dIII), electrophoresed on 1.0% agarose gels and blotted onto positively charged nylon membranes (Amersham Life Science, Buckinghamshire, UK). Hybridization was performed with the DIG Luminescent Detection Kit as recommended by the supplier (Roche). Gene-specific hybridization probes were obtained from the 3′ region of the cDNA, including the 3′-UTR, of *TGDEFA*, *TGDEFB*, and *TGGLO* and labeled with the DIG-High Prime kit (Roche).

Northern blot analysis

Total RNA was isolated from carpels, stamens, inner tepals, outer tepals, leaves, bracts, stems, bulbs and roots by an SDS-phenol method (Kisaka *et al.*, 1996). Total RNA (30 μg) was separated by electrophoresis on 1.2% agarose gels containing 5% formaldehyde and 1× MOPS. The gels were blotted overnight onto positively charged nylon membranes (Amersham) by standard techniques (Sambrook *et al.*, 1989). To avoid cross-hybridization with other members of MADS-box gene family, gene-specific hybridization probes were obtained from the 3′ region of the cDNA, including the 3′-UTR of the *TGDEFA*, *TGDEFB*, and *TGGLO* genes and labeled with the PCR DIG labeling mix as recommended by the supplier (Roche). Hybridization was performed with the DIG Luminescent Detection Kit (Roche).

In vitro *DNA-binding assays*

Plasmids derived from the pSPUTK *in vitro* transcription-translation vector (Stratagene) to produce AP3 and PI proteins have been described previously (Riechmann *et al.*, 1996). The inserts had been subcloned into pSPUTK via PCR.

In case of full-length *TGDEFA* and *TGDEFB*, the *Bam*HI site of pSPUTK was used, while in case of full-length *TGGLO*, the *Nco*I-*Bam*HI site was used. All relevant regions were sequenced after cloning on both strands as described above to check the correctness of the primary structures.

Proteins were synthesized with the T_NT SP6 coupled reticulocyte lysate system (Promega) according to the manufacturer's instructions. They were always [^{35}S]-methionine-labeled and were produced in the expected sizes and in similar amounts, as tested on 12% SDS-PAGE gels, with which X-ray films were exposed after gel run. Binding assays were carried out using double-stranded oligonucleotides containing CArG-box sequences (5′-GGATTAGGCAATACTTT*CCATTTTT***A***G*TAACT-3′) derived from the *Arabidopsis* AP3 promoter (CArG-box sequences are in italics; a deviation from the SRE-type CArG-box consensus (CC(A/T)$_6$GG) is highlighted in bold). The DNA fragments were labeled with ^{32}P by Klenow fill-in reactions and purified by PAGE (10% gels) before they were used in DNA-binding experiments.

In vitro translated proteins were tested for DNA binding activity by gel retardation assays (also termed electrophoretic mobility shift assays, EMSAs). Binding conditions were as described (Egea-Cortines *et al.*, 1999). Total reaction volumes were always 12 μl, containing about 1 ng of labeled, double-stranded oligonucleotides and roughly the same amounts of proteins. Within an experimental series, aliquots were used except for the components that varied. Reactions were incubated for 20 min on ice and loaded on a running 4% polyacrylamide/bisacrylamide (30:1) gel, which had been pre-run at 100 V in 1× TBE. The gel was run at 150 V. After run, the gel was transferred to paper, dried, and used to expose an X-ray film.

Results

cDNA cloning of DEF- *and* GLO-*like MADS-box genes from* Tulipa gesneriana

We have isolated three RACE cDNA clones, which correspond to three different MADS-box genes from tulip (*Tulipa gesneriana*), at least three times independently. BLAST searches in public databases with the conceptual amino acid sequences of these clones as query sequences revealed that two of these clones share high sequence identity with the maize *SILKY1* (Ambrose *et al.*, 2000) and rice *OSMADS16* genes (Moon *et al.*, 1999). This suggests that these two clones represent *DEF*-like genes from tulip. The other clone revealed high sequence identity with the rice *OSMADS2* and *OSMADS4* genes (Chung *et al.*, 1995) and the maize *ZMM16* and *ZMM18* genes (Münster *et al.*, 2001), indicating that this clone represents a *GLO*-like gene. The 5′ regions corresponding to the cDNAs were isolated by the 5′-RACE method, and cDNA clones comprising the complete coding regions ('full-length clones') were isolated by PCR.

Phylogeny reconstructions with other published MADS-box genes corroborated the view that two of the tulip genes represented by the isolated cDNAs fall into the subfamily (clade) of *DEF*-like genes, and one into the clade of *GLO*-like genes (subfamilies as defined elsewhere; Theissen *et al.*, 1996, 2000). Accordingly, we have named the respective genes *TGDEFA*, *TGDEFB* and *TGGLO*, respectively (Figure 2). Within each clade, these genes are especially closely related to the other monocot MADS-box genes present (Figure 2). Phylogeny reconstruction also showed that *TGDEFA* and *TGDEFB* are more closely related to

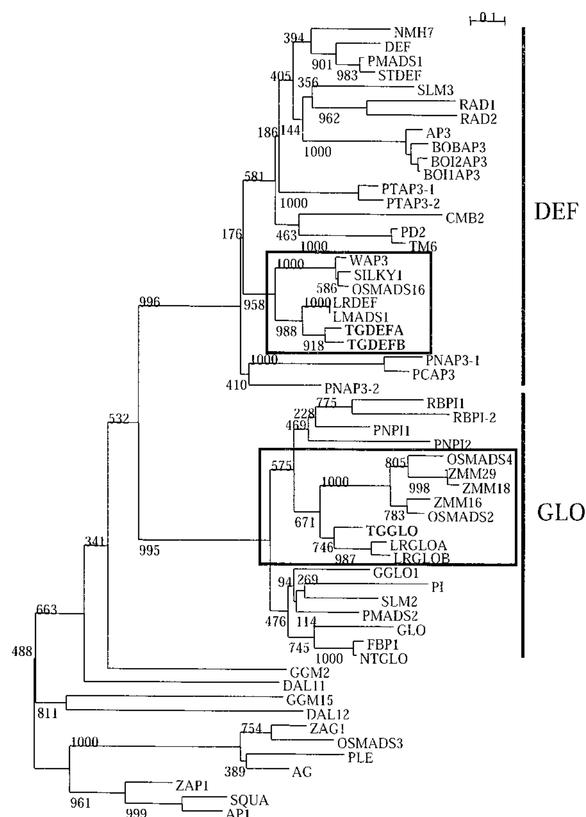

Figure 2. Phylogeny reconstruction of MADS-box genes which belong to the *DEF* and *GLO* subfamilies. This neighbor-joining tree was generated by Clustal W. The numbers next to the nodes give bootstrap values from 1000 replicates. Tulip class B genes are shown in bold and monocot class B genes are framed.

each others than any of these genes is to *LRDEF* and *LMADS1*, the lily *DEF*-like genes (Figure 2). This strongly suggests that the gene duplication which generated *TGDEFA* and *TGDEFB* occurred after divergence of the lineages that led to the extant genera *Lilium* and *Tulipa*.

Analysis of the sequences showed that the *TGDEFA* cDNA is 1016 bp long and encodes a protein with a predicted length of 228 amino acids. The *TGDEFB* cDNA is 1002 bp long and encodes a protein of 231 amino acids. The *TGGLO* cDNA is 876 bp long and encodes a protein of 211 amino acids. The amino acid sequences of *TGDEFA* and *TGDEFB* show an identity of 90%. Both *TGDEFA* and *TGDEFB* have PI-motif-derived regions and paleo AP3 motifs (Figure 3A), while *TGGLO* has only a PI motif (Figure 3B; motifs as defined by Kramer *et al.*, 1998).

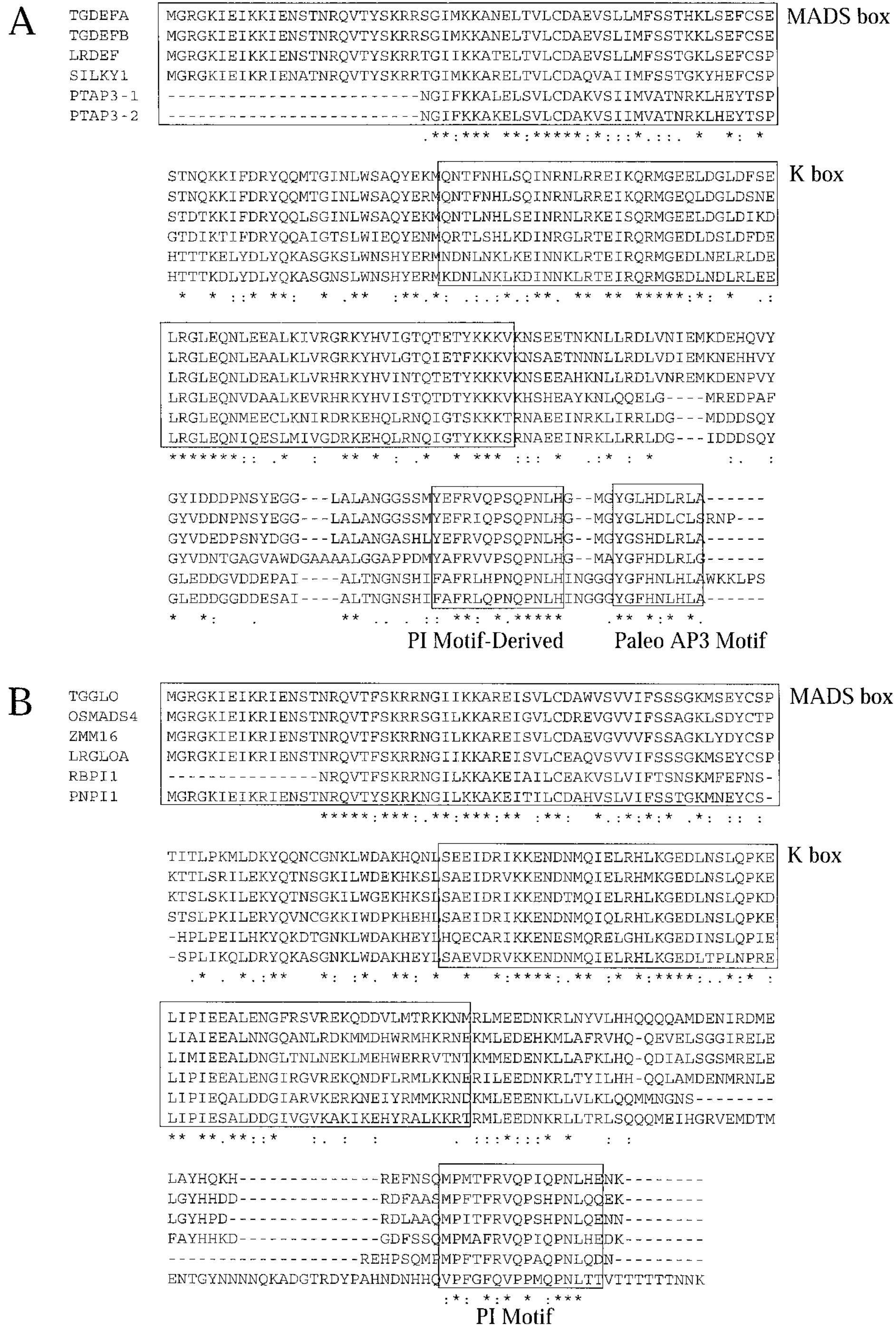

Figure 3. A. Comparison of amino acid sequences among several DEF-like proteins. MADS domains and K domains are shown in boxes. The tulip DEF-like proteins (TGDEFA and TGDEFB) have a PI-motif-derived region and a paleo AP3 motif (Kramer *et al.*, 1998); LRDEF (*Lilium*), SILKY1 (*Zea*), PTAP3-1 and PTAP3-2 (*Pachysandra*). B. Comparison of amino acid sequences among several GLO-like proteins. MADS domains and K domains are shown in boxes. The tulip GLO-like protein (TGGLO) has a PI-motif (Kramer *et al.*, 1998); OSMADS4 (*Oryza*), ZMM16 (*Zea*), LRGLOA (*Lilium*), RBPI1 (*Ranunculus*) and PNPI1 (*Papaver*).

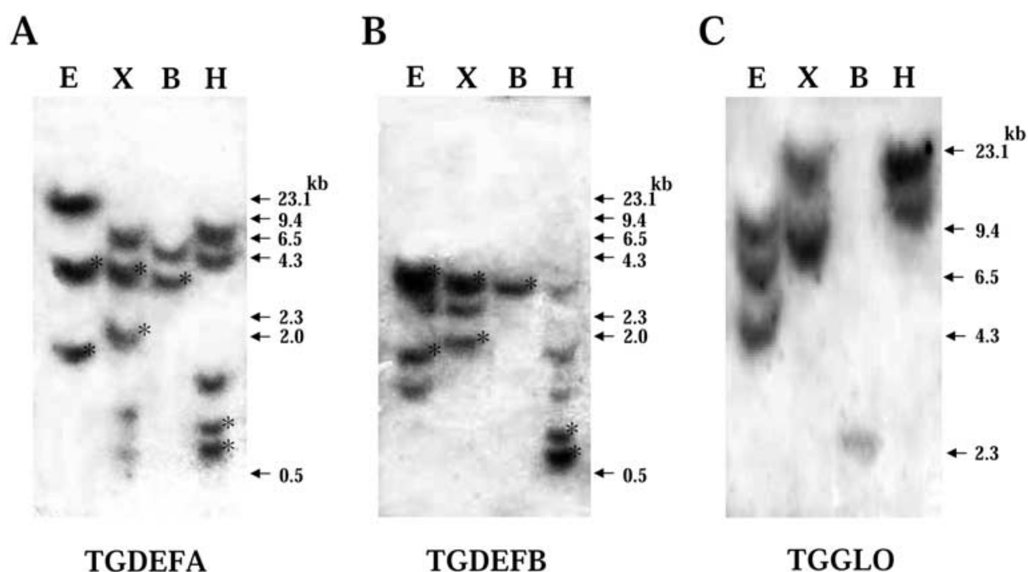

Figure 4. Southern blot analysis of *TGDEFA* (A), *TGDEFB* (B) and *TGGLO* (C) in tulip. Each lane contains 50 μg of total DNA digested with one of four restriction enzymes, *Eco*RI (E), *Xba*I (X), *Bam*HI (B) and *Hin*dIII (H), as indicated above the lanes. Dots mark bands representing cross-hybridization between *TGDEFA* and *TGDEFB* probes.

Genomic organization

To determine the genomic organization of the *TGDEFA*, *TGDEFB*, and *TGGLO* genes in tulip, DNA gel blot ('Southern blot') analyses were performed. Genomic DNA was digested with *Eco*RI, *Xba*I, *Bam*HI, or *Hin*dIII, and hybridized with gene-specific probes. One to five hybridizing bands were found in each lane with *TGDEFA* and *TGDEFB* probes, and some of them showed nearly identical band patterns which are probably caused by cross-hybridization. Our data suggest that, in addition to *TGDEFA* and *TGDEFB*, there may be one or two additional *DEF*-like genes in the tulip genome (Figure 4) Two or three hybridizing bands were found in each lane with a *TGGLO* probe, indicating that there are two or three very similar *GLO*-like genes in the tulip genome (Figure 4).

Expression patterns

Expression of class B genes in *Arabidopsis* and *Antirrhinum* is known to be flower-specific (Jack *et al.*, 1992; Schwarz-Sommer *et al.*, 1992; Tröbner *et al.*, 1992; Goto and Meyerowitz, 1994). To investigate whether the expression of putative tulip class B genes is also flower-specific, we performed RNA gel blot ('northern blot') analyses with total RNA isolated from several organs of tulip. As shown in Figure 5, mRNAs of *TGDEFA* and *TGDEFB* are present in floral organs, especially in whorls 1, 2 and 3 (outer and inner tepals and stamens), but not in any vegetative tissue analyzed even after long exposures. *TGGLO* is strongly expressed in both types of tepals and stamens, and weakly in carpels, leaves, stems and bracts.

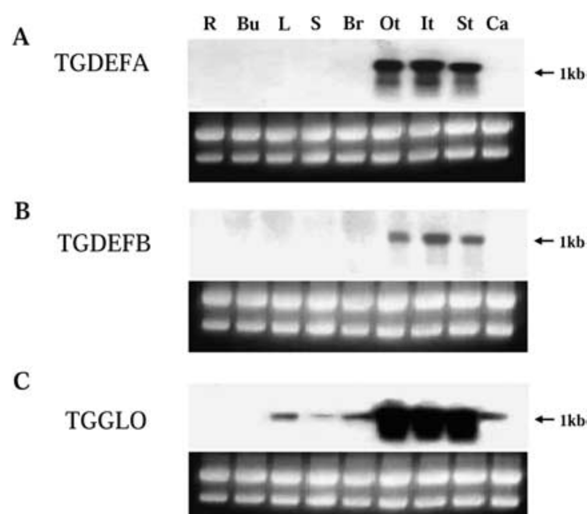

Figure 5. Northern hybridization of *TGDEFA* (A), *TGDEFB* (B) and *TGGLO* (C) in tulip. RNA accumulation in root (R), bulb (Bu), leaf (L), stem (S), bract (Br), outer tepal (Ot), inner tepal (It), stamen (St), and carpel (Ca).

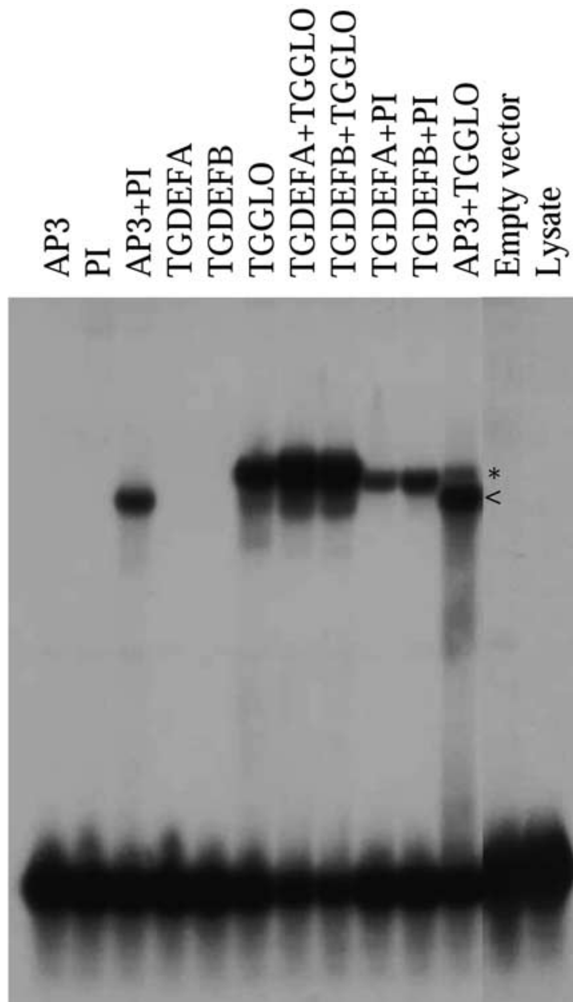

Figure 6. Class B proteins of tulip are able to bind CArG-box DNA sequence elements as homo- and as heterodimers. DNA binding of *in vitro* translated class B proteins as revealed by gel retardation assays is shown. 'Empty vector' symbolizes negative controls where the expression vector did not contain a cDNA insert, but all other experimental conditions were identical to the reactions where *in vitro* translated MADS-domain proteins were generated. 'Lysate' indicates an additional negative control where unprogrammed reticulocyte lysate was incubated with the probes. *marks a band representing an TGGLO homodimer and < indicates an AP3-TGGLO heterodimer.

Protein dimerization and DNA binding

Similar to AP3 from *Arabidopsis* and DEF from *Antirhinum*, tulip DEF-like proteins, TGDEFA and TGDEFB, do not bind to a CArG-box DNA probe without an appropriate heterodimerization partner (Figure 6). In contrast, the tulip GLO-like protein, TGGLO, is able to bind to the CArG probe alone (Figure 6). DNA binding is also observed when TGDEFA/TGGLO or TGDEFB/TGGLO are co-translated. We could detect only one band in the lanes of TGDEFA/TGGLO or TGDEFB/TGGLO, but these bands are thicker than that in the lane of TGGLO alone, indicating that TGDEFA and TGDEFB bind to the CArG probe as heterodimers with TGGLO. DNA binding is also observed when TGDEFA/PI or TGDEFB/PI are co-translated. Since TGDEFA and TGDEFBalone as well as PI alone are not able to bind the probe, one has to postulate a heterodimer between the tulip DEF-like proteins, TGDEFA or TGDEFB, as one partner, and the *Arabidopsis* GLO-like protein, PI, as the other partner. DNA binding is also observed when TGGLO is co-translated with AP3 (Figure 6). In this case, two bands with similar but not identical gel electrophoretic mobility are visible, representing a TGGLO homodimer and a TGGLO-AP3 heterodimer, respectively (Figure 6).

Discussion

We have isolated *DEF*- and *GLO*-like genes, i.e. putative class B genes, from tulip. DEF-like proteins of higher eudicots, lower eudicots and magnolid dicots have different conserved motifs in their C-terminal region. Higher eudicots have an euAP3 motif, whereas lower eudicots and magnolid dicots have a paleoAP3 motif (Kramer *et al.* 1998). TGDEFA and TGDEFB have paleoAP3 motifs as have SILKY and OSMADS16, putative DEF orthologues from maize and rice, respectively (Moon *et al.*, 1999; Ambrose *et al.*, 2000). We have isolated *DEF*-like genes from two other monocots, lily (*Lilium regale*) and asparagus (*Asparagus officinalis*) (Winter *et al.*, 2002; Park *et al.*, 2003). Their proteins also have a paleoAP3 motif in the C-terminal region. This suggests that all of the monocot DEF-like proteins have paleoAP3 motifs, which nicely fits to the phylogenetic position of monocots as basal angiosperms somewhere nested within magnolid dicots (Soltis *et al.*, 1999). Exclusive presence of the paleoAP3 motif in monocots is compatible with the hypothesis that the euAP3 motif is synapomorphic for a clade of DEF-like proteins which is restricted to higher eudicots (Kramer *et al.*, 1998).

In monocotyledonous plants, floral B-function genes were isolated from several grass plants. *SILKY1* is a *DEF*-like gene, and *ZMM16*, *ZMM18* and *ZMM29* are *GLO*-like genes from maize. The sequences and expression patterns of these genes and the nature of the homeotic conversions in *si1* mutants support the idea

that these genes are B-functional genes from maize (Ambrose *et al*., 2000; Münster *et al*., 2001). In addition, putative *GLO* orthologues, *OSMADS4* and *OSMADS2*, and a putative *DEF* orthologue, *OSMADS16*, have been identified in rice (Chung *et al*., 1995; Kang *et al*., 1998; Moon *et al*., 1999). The expression patterns of these genes and the co-suppression phenotype of *OSMADS4* nicely fit into an ABC model (Kang *et al*., 1998; Kyozuka *et al*., 2000; Lee *et al*., 2000). These findings indicate that at least some aspects of the B function are conserved between grasses (monocots) and eudicots. These aspects of the B function must have been present in the most recent common ancestor of the grasses and the eudicots, which was also the most recent common ancestor of the eudicots and the Liliaceae (monocots). So we expect these aspects of the B function also in tulip (Liliaceae).

It appears quite likely that the specification of male reproductive organs, i.e. stamens, by class B genes is among the conserved features of the B function. Whether higher eudicot petals, Liliaceae tepals and grass lodicules are homologous organs is controversial (Baum and Whitlock 1999; Ambrose *et al*., 2000; Kramer and Irish, 2000). It cannot be taken for granted, therefore, that *DEF*- and *GLO*-like genes are involved in the specification of the typical tepals of many Liliaceae species. That petaloid organs other than those of the second floral whorl of higher eudicots obtain their petaloidy via a transference of the B function has been suggested, including cases within eudicots (e.g. petaloid sepals in *Clermontia* and *Impatiens*) (Albert *et al*., 1998; Baum and Whitlock, 1999; Theisssen *et al*., 2000). Transference of B function is also the basic idea to explain the petaloid appearance of tulip outer tepals, as suggested by the modified ABC model (van Tunen *et al*., 1993; Theissen *et al*., 2000).

The expression pattern of *TGDEFA* and *TGDEFB* showed that these genes are expressed in floral organs, especially in whorls 1, 2 and 3 (outer and inner tepal and stamen, respectively) but not in any vegetative tissues analyzed. *TGGLO* is strongly expressed in these three organs and weakly in carpels, leaves, stems and bracts. In *Arabidopsis* and *Antirrhinum*, the expression of class B genes persists during emergence of stamen and petal primordia and later during maturation of these organs (Jack *et al*., 1992; Schwarz-Sommer *et al*., 1992; Tröbner *et al*., 1992; Goto and Meyerowitz, 1994). Using the temperature-sensitive *DEF* mutant, Zachgo *et al*. (1995) reported that continual expression is necessary to establish and maintain wild-type petal organ identity. We used a very late stage of flower buds, however, and could detect strong expression in floral organs in tulip. This expression pattern in tulip is consistent with that in *Arabidopsis* and *Antirrhinum*. In *Arabidopsis* and *Antirrhinum*, class B genes are required to specify petal and stamen identities. And, most importantly, ectopic expression of both *PI* and *AP3* genes in *Arabidopsis* resulted in the conversion of sepals into petals and carpels into stamens, demonstrating that these genes are not only required, but also sufficient to provide the B function in flowers (Krizek and Meyerowitz, 1996). Meanwhile we know that a combination of class A, B and E (*SEPALLATA*) genes is sufficient to superimpose petaloid identity even on vegetative organs, at least in *Arabidopsis* (Honma and Goto, 2001; Pelaz *et al*., 2001; Theissen, 2001; Theissen and Saedler, 2001).

Northern hybridization analysis with total RNA of dissected floral organs showed that all the putative tulip class B genes are expressed in outer and inner tepals and stamens (Figure 5). Importantly, both types of class B genes (*DEF*- and *GLO*-like genes), which are sufficient for the B-function in *Arabidopsis*, are expressed in tulip outer tepals. Assuming that *DEF*- and *GLO*-like genes are also sufficient for the B function in tulip, this strongly supports the modified ABC model (Figure 1).

However, an alternative scenario is conceivable. In higher eudicots, the B function is provided by heterodimers of DEF- and GLO-like proteins. *In vitro* DNA-binding assays showed that the combinations of TGDEFA and TGGLO, and TGDEFB and TGGLO also heterodimerize like eudicot DEF- and GLO-like proteins (Figure 6). It is noteworthy, however, that TGGLO can bind to a CArG-box DNA probe also as a homodimer. Homodimerization of GLO-like proteins was also found in lily (LRGLOA and LRGLOB; Winter *et al*., 2002), indicating that this phenomenon might be common in Liliaceae plants, or even in other monocot species, although evidence for homodimerization of GLO-like genes from rice and maize has not been reported yet. At least in tulip and lily, however, it is conceivable that in the outer tepals the B function is provided by homodimers of GLO-like proteins rather than heterodimers of GLO- and DEF-like proteins.

Further insight into the specification of floral organ identity in tulip flowers might be provided by protein and mutant analysis. In the *viridiflora* mutant of tulip, the perianth organs of the outer as well as the inner whorl are greenish and resemble the sepals of higher eudicots, whereas the stamens are transformed

into carpeloid organs (van Tunen *et al.*, 1993). This phenotype could be explained by the modified ABC model simply by a loss of B function. Thus *viridiflora* mutant flowers should be checked for a reduction in *TGDEFA*, *TGDEFB* and *TGGLO* expression as well as mutations in the coding region. Moreover, accumulation of TGDEFA, TGDEFB and TGGLO proteins should be checked by western blot analysis as soon as specific antibodies are available in order to identify possible cases of posttranscriptional gene regulation and to clarify the functional importance of the mRNA expression patterns.

An even better functional and evolutionary understanding of the *DEF*- and *GLO*-like genes from Liliaceae plants may be obtained by transgenic technology. For example, the evolutionary importance of these loci in the transference of the B function might be tested by transferring them (including promoters and enhancers) into relatives with sepaloid outer tepals (such as *Trillium*; Baum, 1998). If these experiments work (difficult as they are), and petaloid tepals develop in the trangenics, this would corroborate the hypothesis that *cis*-regulatory changes within the B-function gene loci are responsible for the heterotopic expression of the *DEF*- and *GLO*-like genes and thus the transference of the B function (Baum, 1998). A comparison of the promoter sequences from *Trillium* and tulip may reveal the critical sequence elements. Alternatively, regulators ('*trans*-acting factors') of the *DEF*- and *GLO*-like genes may have changed during the origin of petaloid outer tepals. In addition, ectopic expression of diverse ABC genes in transgenic tulip plants may provide valuable data about the specification of floral organ identity in lily-like plants.

The expression pattern of *TGGLO* in female reproductive organs (Figure 5C) resembles that of other monocot *GLO*-like genes, such as *OSMADS4* from rice and *ZMM16*, *ZMM18* and *ZMM29* from maize. This suggests that monocot *GLO*-like genes might have some function in female reproductive organs. Heterodimers of TGDEFA/TGGLO or TGDEFB/TGGLO may not exist in female reproductive organs, since *TGDEFA* and *TGDEFB* mRNAs do not accumulate in carpels (Figure 5A, B). It cannot be excluded, however, that the proteins encoded by these genes move from the stamen into the carpel whorl. In any case, TGGLO can homodimerize, suggesting that the TGGLO homodimer might have some functions in female reproductive organs.

Since *TGGLO* is also expressed in some vegetative organs, such as leaves, stems and bracts, it cannot be excluded that the TGGLO homodimer has some function in these organs. Similar observations and conclusions have been made for a *GLO*-like gene from maize (Münster *et al.*, 2001).

Obviously, although the data presented here support the modified ABC model for tulip as suggested by van Tunen *et al.* (1993), much remains to be done to fully understand the floral organ identity genes, very likely including the *DEF*- and *GLO*-like genes, from tulip and other Liliaceae.

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