Journal of Experimental Botany, Vol. 61, No. 1, pp. 75–85, 2010
doi:10.1093/jxb/erp279 Advance Access publication 18 September, 2009
This paper is available online free of all access charges (see http://jxb.oxfordjournals.org/open_access.html for further details)

Journal of Experimental Botany
www.jxb.oxfordjournals.org

RESEARCH PAPER

Functional characterization of B class MADS-box transcription factors in *Gerbera hybrida*

Suvi K. Broholm, Eija Pöllänen*, Satu Ruokolainen, Sari Tähtiharju, Mika Kotilainen[†], Victor A. Albert[‡], Paula Elomaa and Teemu H. Teeri[§]

Department of Applied Biology, PO Box 27, University of Helsinki, FIN-00014 Finland

Received 26 June 2009; Revised 21 August 2009; Accepted 24 August 2009

Downloaded from https://academic.oup.com/jxb/article/61/1/75/566697 by guest on 24 April 2024

Abstract

According to the classical ABC model, B-function genes are involved in determining petal and stamen development. Most core eudicot species have B class genes belonging to three different lineages: the *PI*, eu*AP3*, and *TM6* lineages, although both *Arabidopsis* and *Antirrhinum* appear to have lost their *TM6*-like gene. Functional studies were performed for three gerbera (*Gerbera hybrida*) B class MADS-box genes—*PI*/*GLO*-like *GGLO1*, eu*AP3* class *GDEF2*, and *TM6*-like *GDEF1*—and data are shown for a second eu*AP3*-like gene, *GDEF3*. In phylogenetic analysis, *GDEF3* is a closely related paralogue of *GDEF2*, and apparently stems from a duplication common to all Asteraceae. Expression analysis and transgenic phenotypes confirm that *GGLO1* and *GDEF2* mediate the classical B-function since they determine petal and stamen identities. However, based on assays in yeast, three B class heterodimer combinations are possible in gerbera. In addition to the interaction of GGLO1 and GDEF2 proteins, GGLO1 also pairs with GDEF1 and GDEF3. This analysis of *GDEF1* represents the first functional characterization of a *TM6*-like gene in a core eudicot species outside Solanaceae. Similarly to its relatives in petunia and tomato, the expression pattern and transgenic phenotypes indicate that *GDEF1* is not involved in determination of petal identity, but has a redundant role in regulating stamen development.

Key words: Asteraceae, evo–devo, flower development, organ identity.

Introduction

In core eudicots, the identity of petals and stamens is known to be specified by B class MADS-box genes. The best studied model species, *Arabidopsis* (*Arabidopsis thaliana*) and *Antirrhinum* (*Antirrhinum majus*), have two B genes: *PISTILLATA* (*PI*) and *APETALA3* (*AP3*), and *GLOBOSA* (*GLO*) and *DEFICIENS* (*DEF*), respectively. The two proteins interact to form a heterodimer that is required for their function in DNA binding (Schwarz-Sommer *et al.*, 1992; Tröbner *et al.*, 1992; Goto and Meyerowitz, 1994). Phylogenetic reconstructions show that *PI*/*GLO*- and *AP3*/*DEF*-like genes form separate gene lineages that arose from a duplication event that occurred before the rise of modern angiosperms (Kim *et al.*, 2004; Hernandez-Hernandez *et al.*, 2007). The *AP3*/*DEF* lineage has undergone another duplication event at the base of core eudicots, resulting into two paralogous lineages, eu*AP3* and *TOMATO MADS BOX GENE6* (*TM6*). The eu*AP3* and *TM6* lineage genes encode specific C-terminal motifs, called the euAP3 and paleoAP3 motif (Kramer *et al.*, 1998, 2004; Kramer and Irish, 2000). The novel euAP3 motif seems to have originated from the ancestral paleoAP3 motif via a simple frameshift mutation close to the gene duplication event (Vandenbussche *et al.*, 2003; Kramer *et al.*, 2006). Though the C-terminal motifs are highly conserved within the gene lineages, suggesting a functional importance, their exact function remains unclear (Piwarzyk *et al.*, 2007; Su *et al.*, 2008).

* Present address: Department of Health Sciences, PO Box 35, University of Jyväskylä, FIN-40014, Finland.
[†] Present address: Department of Biological and Environmental Sciences, PO Box 56, University of Helsinki, Finland.
[‡] Present address: Department of Biological Sciences, University at Buffalo (SUNY), Buffalo, NY 14260, USA.
[§] To whom correspondence should be addressed. E-mail: teemu.teeri@helsinki.fi
© 2009 The Author(s).
This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (http://creativecommons.org/licenses/by-nc/2.5/uk/) which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.

Members of the *TM6* lineage are the most poorly studied B class genes as neither *Arabidopsis* nor *Antirrhinum* have *TM6* gene copies (Lamb and Irish, 2003). The most thorough functional studies on *TM6* lineage genes so far have been performed in Solanaceae, on *Petunia hybrida TM6* (*PhTM6*) (Rijpkema *et al.*, 2006) and tomato (*Solanum lycopersicum*) *TM6* (de Martino *et al.*, 2006). The expression patterns of these *TM6* lineage genes differ from those of *PI/GLO* and eu*AP3* lineage genes in that they have a broader expression domain. Their transcripts are detected not only in petals and stamens, but also in carpels and ovules (Vandenbussche *et al.*, 2004; de Martino *et al.*, 2006). The petunia and tomato *TM6* genes are involved in stamen development and not in petal development, while the eu*AP3* genes play a role in both petal and stamen development. The petunia *PhTM6* and *PhDEF* act completely redundantly in stamen development, while the tomato *TAP3* and *TM6* are only partly redundant: the *TAP3* gene has unique functions in stamen development that cannot be fulfilled by *TM6* (de Martino *et al.*, 2006; Rijpkema *et al.*, 2006).

To obtain a better understanding of the functional differentiation of B class MADS-box genes, *PI/GLO*- and *AP3/DEF*-like genes have been analysed in gerbera. Gerbera belongs to the large sunflower (Asteraceae) family, which lies in a lineage of asterids distinct from the position of Solanaceae (Angiosperm Phylogeny Group II, 2003). Typical of Asteraceae, the inflorescence comprises hundreds of tightly packed flowers with specialized structures and functions. Three distinct flower types can be distinguished: the outermost ray and trans flowers have showy petals and non-functional rudimental stamens, whereas the disk flowers in the centre are smaller and hermaphroditic with functional stamens. Three gerbera B class genes that belong to different subfamilies have previously been identified. *GERBERA GLOBOSA-LIKE1* (*GGLO1*) is a *PI/GLO* lineage gene, *GERBERA DEFICIENS-LIKE2* (*GDEF2*) is a eu*AP3* gene, and *GDEF1* is a *TM6*-like gene (Yu *et al.*, 1999). Here functional analyses for these genes are presented and, furthermore, data are shown for a fourth gerbera B gene, *GDEF3*, which appears to be the result of an Asteraceae-specific gene duplication in the eu*AP3* subfamily. The data indicate that the *PI/GLO* and eu*AP3* lineage genes *GGLO1* and *GDEF2*, respectively, function in petal and stamen development and encode the classical B-function in gerbera. An additional novel phenotype, the conversion of petals into ovary wall-like tissues, was also observed when B-function genes were down-regulated. Expression of the *TM6* lineage gene *GDEF1* differs from that of the other gerbera B class genes in several aspects, such as being absent during the early petal primordia initiation. However, transgenic phenotypes do not show a unique function for *GDEF1* but instead suggest it to be mainly redundant with other gerbera B class genes. Functional redundancy is further supported by the yeast two- and three-hybrid assays which show that the gerbera B class proteins form three kinds of heterodimers with parallel multimeric protein interaction capacities.

Materials and methods

Isolation of full-length GDEF3

Based on the expressed sequence tag (EST) sequence of *GDEF3* (*G0000700006A02*), primers for standard 5′ rapid amplification of cDNA ends (RACE) were designed. Ray flower petals (developmental stages 2 and 3; Helariutta *et al.* 1993) were used as starting material and cDNA was synthesized as described in Laitinen *et al.* (2008). Full-length cDNA amplification by RT-PCR (with a forward primer 5′-ATCCAAATCAATGGCGAGAG-3′ and a reverse primer 5′-CCGTCATAATCCAAA-TCAGACA-3′) was performed to ensure that the 5′ fragment originated from the same transcript. The cDNA was cloned into ZeroBlunt vector and sequenced in both directions. The full-length sequence for *GDEF3* has been deposited in GenBank (http://www.ncbi.nlm.nih.gov) under accession number FJ817421.

Phylogenetic analysis

Phylogenetic analyses were performed on corresponding nucleotide and amino acid alignments for the MADS and K domains of selected class B MADS-box factors. Both parsimony and maximum likelihood methods were used on nucleotide and amino acid alignments. Since the analyses were largely congruent, only the maximum likelihood results from the nucleotide sequence alignment are described and presented. For these analyses, the options used with the PHYML (Guindon and Gascuel, 2003) web interface (Guindon *et al.*, 2005) were: (i) a starting tree constructed with the BIONJ (Gascuel, 1997); (ii) best of NNI- and SPR-swapped trees; (iii) a random tree from five data addition sequences; (iv) the HKY85 evolutionary model (Hasegawa *et al.*, 1985); (v) estimated proportion of invariable sites=0.122; (vi) six substitution rate categories; (vii) estimated gamma distribution parameter=1.170; (viii) transition/transversion ratio: 2.883; (ix) empirical base frequencies; (x) tree topology optimization; and (xi) branch length and rate parameter optimization. A total of 500 boostrap resampling replicates were used to estimate support for clades (Felsenstein, 1985).

RNA blot and in situ *hybridizations*

RNA blots were done as previously reported by Broholm *et al.* (2008). The floral organs were collected from ray and disk flowers at different developmental stages (pooled at stages 3, 5, 7, and 9 for ray; and at stages 6 and 8 for disk flowers). The developmental stages of the gerbera inflorescence have been described in Helariutta *et al.* (1993). For gene-specific probes, the 3′ end of the cDNA was used (for *GGLO1*, 217 bp). The plasmid pGGLO1as was digested with *Xho*I and *Bgl*II; for *GDEF2* (423 bp) pHTT664.3 with *Eco*RI and *Bam*HI; for *GDEF3* (335 bp) G0000700006A02 with *Sac*II and *Kpn*I; and for *GDEF1* (283 bp) pHTT661.2 with *Eco*RI and *Bam*HI. *In situ* hybridization analyses using gene-specific probes were performed as in Elomaa *et al.* (2003). Probe concentration for *GGLO1*, *GDEF2*, and *GDEF3* was 0.4 μg ml^{-1} kb^{-1}, and for *GDEF1* 0.5 μg ml^{-1} kb^{-1}. Detection time was 16 h for *GGLO1* and *GDEF2*, and 40–45 h for *GDEF3* and *GDEF1*.

Plant transformation and analysis of transgenic lines

Agrobacterium-mediated transformation of the full-length gene constructs 35S-*GGLO1*, 35S-*GDEF2*, and 35S-*GDEF1* into gerbera was performed as previously described (Elomaa and Teeri, 2001). Integration of the transgene was verified using standard DNA hybridization. Scanning electron microscopy (SEM) analysis of the transgenic flower organs was performed as described by Uimari *et al.* (2004).

Yeast assays

Full-length gerbera B class MADS-box cDNAs (*GGLO1*, *GDEF1*, *GDEF2*, and *GDEF3*) were introduced into the Gateway system

Downloaded from https://academic.oup.com/jxb/article/61/1/75/566697 by guest on 24 April 2024

using PCR (PCR Cloning System with Gateway Technology with pDONR221, Invitrogen). Primers flanking the first methionine of the gene and the stop codon were designed according to Invitrogen's instructions. Two nucleotides were added between the *attB1* sequence and the start codon. The Gateway primer sequences are shown in Supplementary Table S2 available at *JXB* online. The PCRs were run according to the guidelines of the Phusion DNA polymerase (Finnzymes). The PCR products were polyethylene glycol (PEG) purified and recombined to pDONR221 plasmid to create the Gateway entry clones, according to Invitrogen's instructions.

The entry clones were recombined into the activation domain- and binding domain-containing plasmids pDEST22 and pDEST32 (Invitrogen). The pDEST22 and pDEST32 derivatives carrying the gerbera B class MADS-box genes were transformed into both yeast strains PJ69-4A and PJ69-4α (James *et al.*, 1996). The pDEST32 clones containing N-terminal binding domain fusions were tested for autoactivation by plating them on the yeast medium SD lacking adenine (SD –Ade) and histidine (SD –His), and supplemented with 1, 5, 10, 25, and 50 mM 3-amino-1,2,4-triazole (3-AT) (Sigma A8056). To obtain yeast double transformants, the A and α types of yeast strains were mated by pipetting them on top of each other on SD Complete plates containing all the essential amino acids. Yeast double transformants were plated on selection plates SD –Leu –Trp, and these colonies were replica plated on YPAD, SD –Leu –Trp –Ade, and SD –Leu –Trp –His + 25 mM 3-AT essentially as described in the ProQuest Two-Hybrid System Manual (Invitrogen PQ10001-01). X-gal assay was performed for yeast cells grown on YPAD plates according to Invitrogen's instructions.

Results

Phylogenetic analysis of the gerbera B class MADS-box genes

In addition to the three previously isolated gerbera B class genes (*GDEF1*, *GDEF2*, and *GGLO1*), a fourth gene, named *GDEF3*, was identified by EST sequencing from a gerbera cDNA library that was constructed from early stages of petal development (Laitinen *et al.*, 2005). Maximum likelihood phylogenetic analyses demonstrated that, in general, the B class genes from Asteraceae seem to form specific subgroups within the three B class clades (*PI*/*GLO*, eu*AP3*, and *TM6*) found in core eudicots (Fig. 1).

In the eu*AP3* lineage the Asteraceae-specific subgroup is further divided into two paralogous groups, in which *GDEF2* and *GDEF3* have putative orthologues from both sunflower (*Helianthus annuus*) and chrysanthemum (*Dendranthema grandiflorum*) (Fig. 1). This indicates that the eu*AP3* lineage has undergone a gene duplication event early in the Asteraceae lineage. *GDEF2* and *GDEF3* both encode the typical eu*AP3* C-terminal motifs. Alignment of the inferred amino acid sequences of the selected eu*AP3* lineage members (Supplementary Fig. S1 at *JXB* online) showed several amino acid changes that were specific to the GDEF2/HAM63/CDM115 and GDEF3/HAM2/CDM19 groups: three were observed in the K-domain and three in the C-terminal region.

Interestingly, the gerbera *TM6* lineage gene *GDEF1* encodes a paleo*AP3* C-terminal motif (YELHDHQHTN) that is highly diverged from the consensus sequence (YGxHDLRLA) (alignment shown, for example, in Kim *et al.*, 2004). The paleo*AP3* motif of its putative sunflower orthologue *HAM91* (YEPHGLRLD) is much more similar to the consensus paleo*AP3* motif than is that of *GDEF1*.

Expression of B class genes in gerbera tissues

Expression of the gerbera B class genes was studied by RNA blotting and in situ hybridization. Both *GGLO1* and *GDEF1* were found to be expressed exclusively in floral tissues, whereas *GDEF2* and *GDEF3* transcripts were also detected (although only weakly) in vegetative leaves and petioles (Fig. 2). *GGLO1*, *GDEF2*, and *GDEF3* all showed the strongest expression in petals and stamens. *GDEF2* and *GDEF3* also showed a weak signal in pappus bristles (whorl one) and carpel (stigma and style) (Fig. 2). Only *GGLO1* expression was sharply restricted to petals and stamens during organ differentiation (Figs 2 and Fig. 3F, K, P, U), whereas *GDEF2* and *GDEF3* had broader expression domains (Fig. 2).

While *GGLO1*, *GDEF2*, and *GDEF3* showed similar expression in both ray and disk flowers (Fig. 2), the expression pattern of *GDEF1* varied between the two flower types. In disk flowers, *GDEF1* transcripts were detected in all four floral whorls, whereas in ray flowers the expression could not be detected in petals at comparable developmental stages (Fig. 2). The expression of *GDEF1* was detected only very weakly at early stages of ray flower petal development (stages 1–3, data not shown), in contrast to the more consistent expression in disk flower petals (Supplementary Fig. S2 at *JXB* online). All the four gerbera B class genes were expressed in the non-functional stamen rudiments of ray and trans flowers (Fig. 3K, L, M, N for ray flowers, data not shown for trans flower stamens). This suggests that they are not involved in the sex determination of the gerbera flower types.

In situ hybridizations showed further differences in the pattern of *GDEF1* expression in comparison with the other gerbera B class genes. *GDEF1* transcripts were detected in stamen primordia but not in the early petal primordia (Fig. 3D, I), while the expression of *GGLO1*, *GDEF2*, and *GDEF3* was activated in both petal and stamen primordia (Fig. 3A, B, C). Later on, *GDEF1* was expressed in all four whorls, but in spatially restricted patterns. *GDEF1* transcripts were localized in lateral edges of petals in all three flower types (Fig. 3N for ray, S for trans, and X for disk flowers). Thus, the expression of *GDEF1* clearly differs from the uniform pattern of *GGLO1, GDEF2*, and *GDEF3* expression in petals.

In disk flower stamens, *GDEF1* expression was restricted to the adaxial side (Fig. 3X) and, later in development, to the sterile connective tissue (data not shown). However, RNA blots revealed that *GDEF1*, together with *GGLO1*, was expressed in disk flower stamens at later stages than the eu*AP3* genes *GDEF2* and *GDEF3* (Supplementary Fig. S2 at *JXB* online). An additional difference in the *GDEF1* expression pattern was strong expression at late stages of

Downloaded from https://academic.oup.com/jxb/article/61/1/75/566697 by guest on 24 April 2024

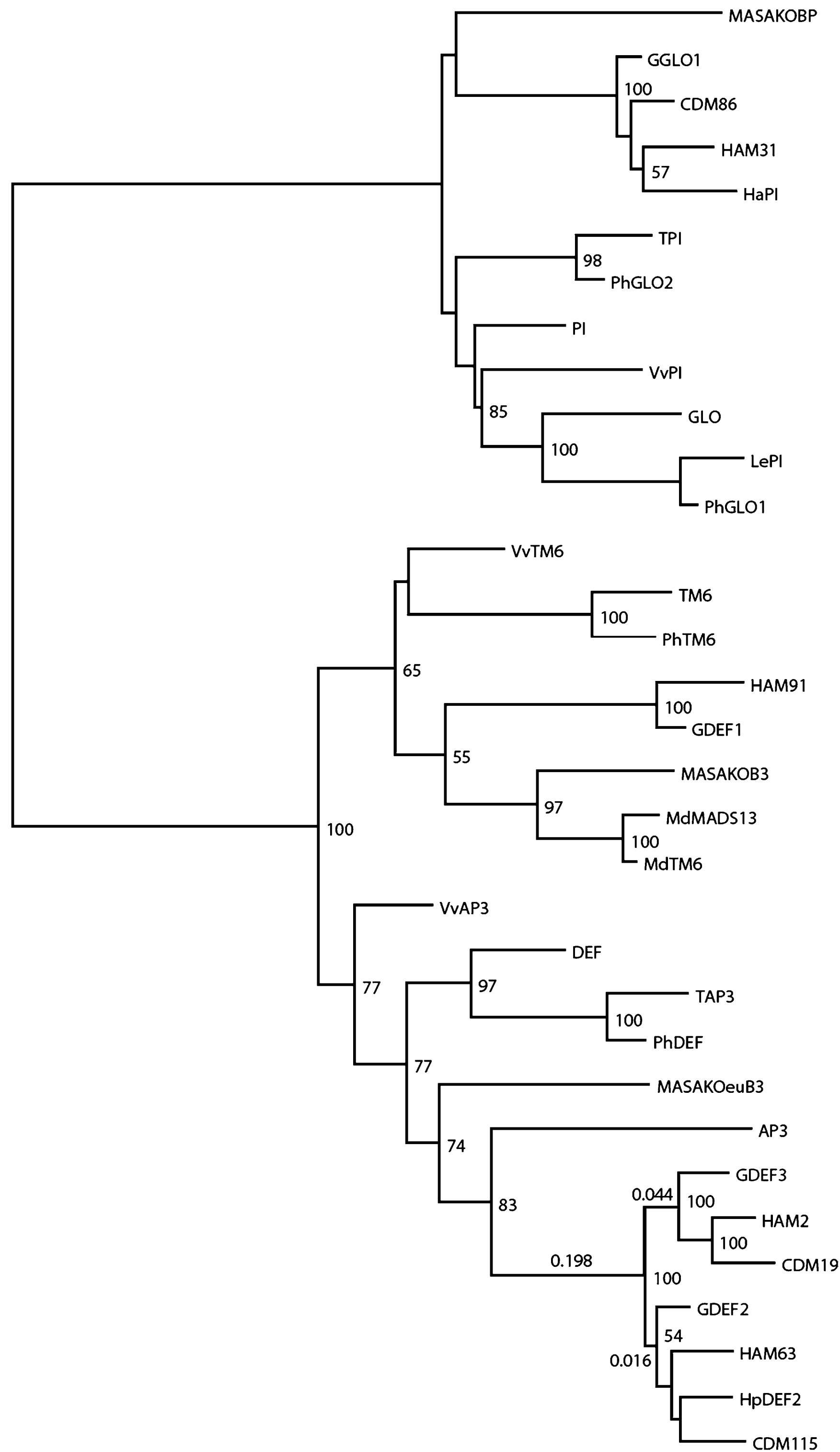

Fig. 1. Phylogenetic analysis of selected B class MADS-box genes based on a nucleotide sequence alignment (~550 bp from the start codon) and the maximum likelihood method. The four gerbera B class genes are located on distinct branches of the tree: *GDEF2* and *GDEF3* in the eu*AP3* clade, *GDEF1* in the *TM6*-like clade, and *GGLO1* in the *PI/GLO* clade. Genes from Asteraceae form two paralogous subgroups within the eu*AP3* clade, the *GDEF3*-like and *GDEF2*-like clades. Bootstrap values at nodes represent percentages of times clades appeared in 500 resampling replicates. Lengths for three key branches (in subsititutions per site) among the Asteraceae genes are also indicated for scale. In addition to gerbera, genes were selected from the following species: *Antirrhinum majus* (GLO, DEF); *Arabidopsis thaliana* (PI, AP3); *Dendranthema grandiflorum* (CDM86, CDM115, CDM19); *Helianthus annuus* (HaPI, HAM31, HAM91, HAM2, HAM63); *Hieracium piloselloides* (HpDEF2); *Malus domestica* (MdTM6, MdMADS13); *Petunia hybrida* (PhGLO1, PhGLO2, PhTM6, PhDEF); *Rosa rugosa* (MASAKOBP, MASAKOB3, MASAKOeuB3); *Solanum lycopersicum* (LePI, TPI, TM6, TAP3); and *Vitis vinifera* (VvPI, VvTM6, VvAP3). GenBank accession numbers are presented in Supplementary Table S1M at *JXB* online.

Downloaded from https://academic.oup.com/jxb/article/61/1/75/566697 by guest on 24 April 2024

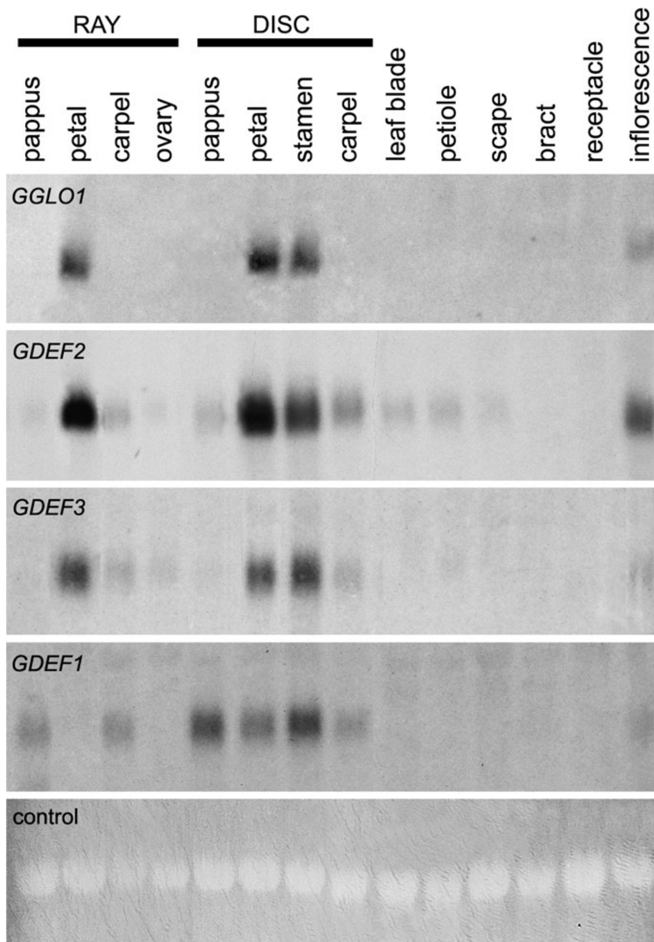

Fig. 2. The expression of gerbera B class genes in floral organs of ray and disk flowers and other tissues. The expression of *GGLO1* is restricted to petals and stamens. *GDEF2* and *GDEF3* are expressed, in addition to petals and stamens, in other floral organs and vegetative tissues. *GDEF1* is expressed in all four floral organs in disk flowers, but in ray flowers the expression was not detected in petals. The lowest panel shows ethidium bromide-stained rRNA bands to control RNA loading.

carpel development when *GDEF2* and *GDEF3* were no longer expressed (Supplementary Fig. S2).

Down-regulation of GGLO1 *and* GDEF2 *expression leads to stamen and petal defects*

In a previous study, the phenotypic effects of constitutive and reduced expression of *GGLO1* were reported (Yu *et al.* 1999). The lines with reduced *GGLO1* expression described in Yu *et al.* (1999) showed homeotic changes in petals but not in stamens. An additional *GGLO1* co-suppression line that shows a stronger phenotype has now been obtained (Tr15, Fig. 4B). In contrast to the previous studies, the identity of both petals and stamens was affected. In accordance with the predicted homeotic changes in B class mutants, stamens were converted into carpeloid structures with carpel-like cells on their surfaces (Fig. 5B). Petals had two different kinds of epidermal trichomes on their abaxial surfaces. On the basal petal tube, trichomes similar to those found in wild-type ovary wall were observed (Fig. 5E). On the distal petal parts (the ligule), there were trichomes that resembled pappus bristles (Fig. 5H). *GGLO1* expression was highly reduced in young inflorescences of the Tr15-line. However, the expression of *GDEF1*, *GDEF2*, and *GDEF3* appeared unaltered (Supplementary Fig. S3 at *JXB* online).

The transgenic lines in which *GDEF2* expression was suppressed also showed phenotypic changes in whorls two and three (Supplementary Fig. S4 at *JXB* online), though their phenotypes were different from those of the *GGLO1* down-regulation lines. Conversion of the basal petal tube of ray flowers into an ovary-like structure was even more striking in the *GDEF2* down-regulation lines, and three new cell types were observed on the petal epidermis (Fig. 6). The basalmost tubular region of the petals had converted to a tissue covered by similar trichomes to those in wild-type ovary wall. Inside this ovary-like structure there was no ovule but instead what appeared to be normal petal tissue. Above the ovary wall-like tissue, a second round of pappus hairs developed. These emerged from round-shaped cells that were similar to cells from which pappus hairs emerge in the wild type. In addition to the alterations in petals, homeotic conversion of stamens towards carpeloid structures was observed (Supplementary Fig. S4). The homeotic conversion of stamens was seen in only one of the lines (Tr22, Fig. 4C), which showed strongest reduction in *GDEF2* expression, and also showed reduction in the expression of *GGLO1* (Supplementary Fig. S3). In the other lines, there were no obvious homeotic changes in stamens. However, disk flower stamens showed earlier senescence by turning brown and dry already at anthesis. The stamens produced some pollen but did not release them readily.

Down-regulation of GDEF1 *expression leads to mild phenotypic effects*

In both of the *GDEF1* antisense lines in which *GDEF1* expression was reduced (Fig. 4D, Supplementary Fig. S3 at *JXB* online), developmental changes in petals and stamens were observed. These changes resembled the mildest phenotypes of the transgenic lines with reduced *GDEF2* or *GGLO1* expression. However, expression of *GDEF2*, *GDEF3*, or *GGLO1* in the young inflorescences of these lines was not altered compared with the wild type (Supplementary Fig. S3). Both stereomicroscopic and SEM analysis showed the presence of few ovary wall-like cells in the abaxial side of ray flower petal tubes (data not shown). No pappus bristle-like hairs were observed. Disk flower stamens were similarly brownish at anthesis and unable to release pollen, as described above for the stamens of the mild anti-*GDEF2*-lines. No obvious homeotic conversions were observed in these stamens (data not shown).

Overexpression of the gerbera B class genes

It was demonstrated previously that transgenic lines expressing *GGLO1* ectopically have clear homeotic conversions (Yu

Downloaded from https://academic.oup.com/jxb/article/61/1/75/566697 by guest on 24 April 2024

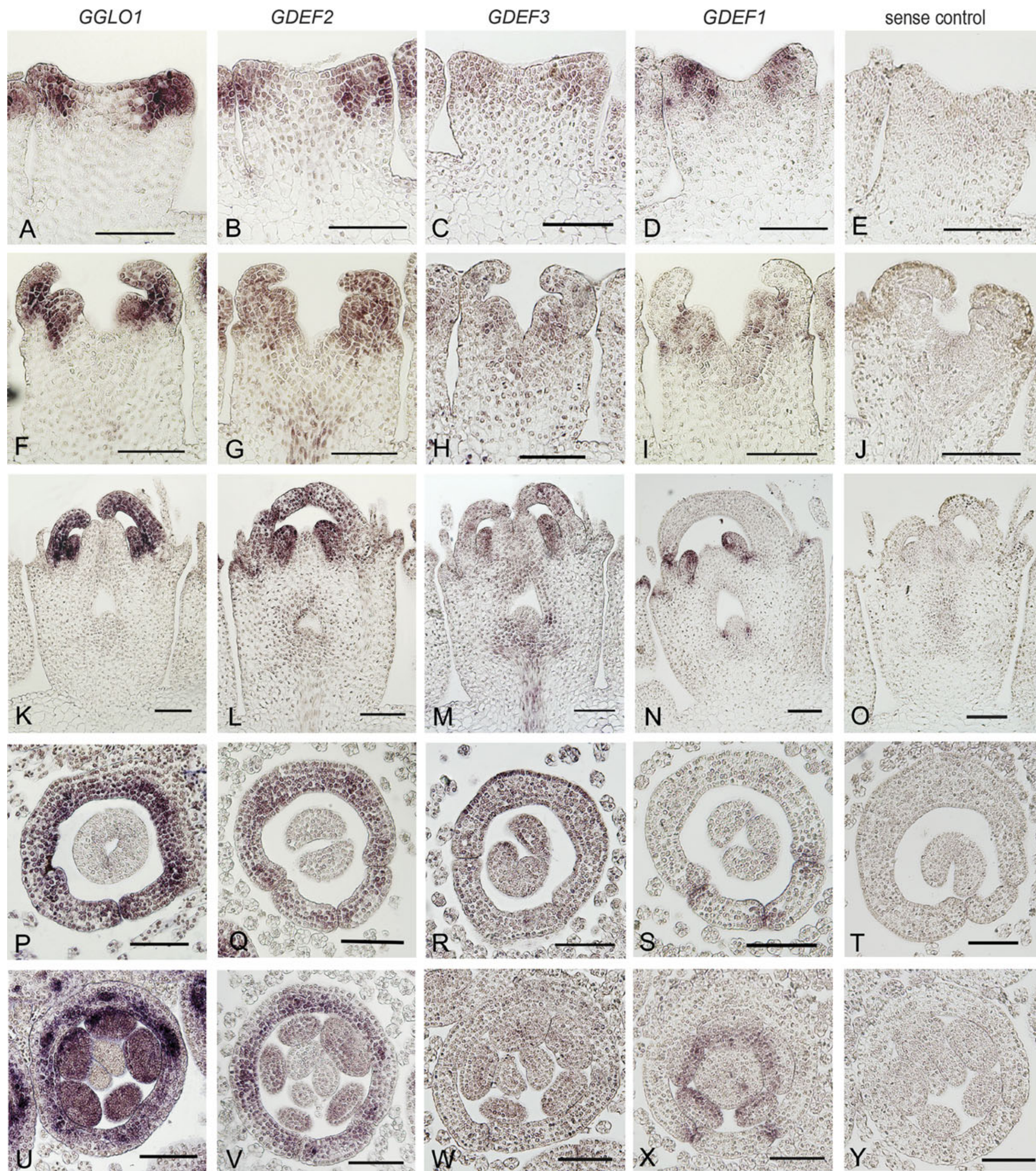

Fig. 3. *In situ* analysis of gerbera B class gene expression in floral primordia at stage 2 (A–E), stage 3–4 (F–J), ray flowers (K–O), trans flowers (P–T), and disk flowers (U–Y). *GGLO1*, *GDEF2*, and *GDEF3* are mainly restricted in petals (pe) and stamens (st). *GDEF1* is not detected in early petal primordia (D, I), but later in a spatially restricted pattern in petals (N, S, X), stamens (N, X), and ovule (N). *GDEF1* also shows early expression in whorl one (w1) (D, I). Negative controls hybridized with a sense probe are shown in the rightmost panel. A–O represent longitudinal sections and P–Y cross-sections. Scale bars, 100 μm.

et al., 1999). Ectopic expression of *GGLO1* caused whorl one pappus bristles to gain petal-like characters, whereas in whorl four, carpels obtained stamen-like traits (Yu *et al.* 1999). In contrast, the six lines expressing *GDEF2* ectopically and five lines expressing *GDEF1* ectopically (data not shown) did not show any phenotypic changes compared with the wild type. The expression of *GDEF2*, *GDEF3*, *GDEF1*, and endogenous *GGLO1* in the 35S promoter-driven *GGLO1* overexpression lines was studied (Supplementary Fig. S5 at *JXB* online). The expression of *GDEF2* was highly up-regulated in whorls one and four as well as in ovaries and leaves. *GDEF3* expression was most clearly up-regulated in stamens and whorl four stamenoid carpels, whereas in petals the expression was unaltered. *GDEF1* expression was enhanced only in whorl four, and, interestingly, its expression was reduced in the whorl one organs that had petal-like characteristics. The

Downloaded from https://academic.oup.com/jxb/article/61/1/75/566697 by guest on 24 April 2024

Fig. 4. The wild-type gerbera inflorescence (A) and transgenic inflorescence phenotypes caused by co-suppression of *GGLO1* expression (B, tr15), co-suppression of *GDEF2* (C, tr22), and reduction of *GDEF1* expression (D, tr12).

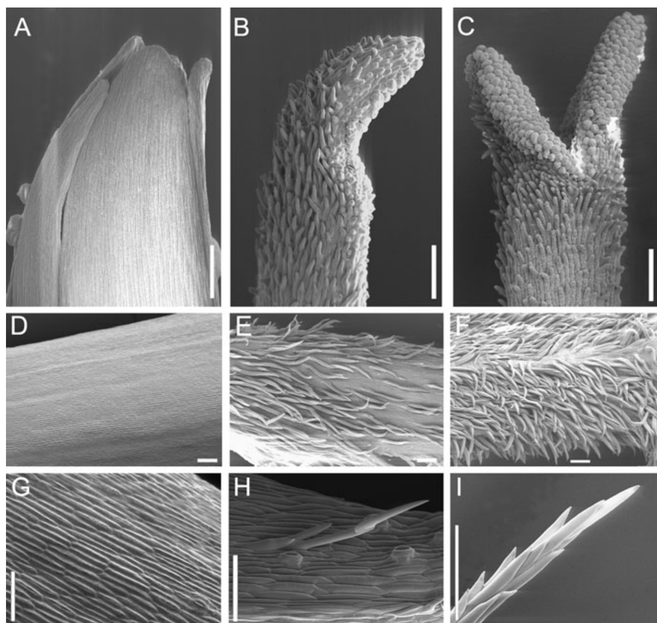

Fig. 5. SEM analysis comparing the epidermal cell structure of gerbera floral organs between the wild type (wt) and transgenic line (tr15) with reduced *GGLO1* expression. Wt stamen (A) and tr stamen (B), which resembles wt carpel (C). Wt petal tube (D) and tr petal tube (E) with trichomes similar to trichomes in the wt ovary wall (F). Wt petal ligule (G) and tr petal ligule (H) with trichomes that resemble wt whorl one pappus bristles (I). Scale bars, 100 μm.

endogenous *GGLO1* expression was also up-regulated in these lines and independent of the floral context (Supplementary Fig. S5). In general, overexpression of *GGLO1* led to up-regulation of *GDEF2*, *GDEF3*, and *GDEF1* expression and caused homeotic conversions. In contrast, overexpression of *GDEF2* did not alter the expression of *GGLO1* (data not shown). The inability of *GDEF2* to induce ectopic *GGLO1* expression might relate to the strictly restricted expression of *GGLO1* in wild-type whorls two and three, and this in turn may explain the lack of phenotypic changes in the *GDEF2* overexpression lines.

Protein interactions of the gerbera B class MADS domain proteins

Using GAL4 yeast two-hybrid assays, it could be shown that the GGLO1 protein can form a heterodimer with all the other three gerbera B class proteins GDEF1, GDEF2, and GDEF3 (Supplementary Fig. S6 at *JXB* online). The interaction capacity of the B class proteins (except GDEF3) was tested with 11 other gerbera AGAMOUS-, SQUAMOSA-, or SEPALLATA3-like MADS domain proteins (S Ruokolainen *et al.*, unpublished results). The B class proteins did not interact with any other class of MADS domain proteins in the pairwise interaction assays. Further, no homodimers or interactions between the eu*AP3* or *TM6* clade members were observed.

Downloaded from https://academic.oup.com/jxb/article/61/1/75/566697 by guest on 24 April 2024

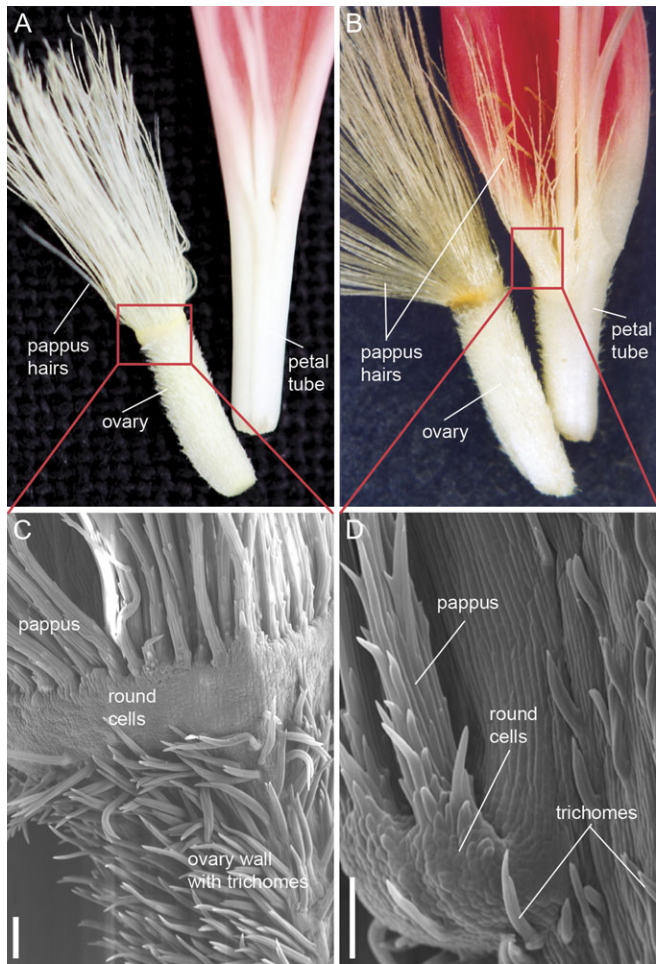

Fig. 6. Comparison of the wild type (A, C) and transgenic line (tr22) with reduced *GDEF2* expression (B, D). Transgenic petals had obtained three new cell types. The petal tube was covered by similar trichomes to those in ovary walls (B, D). In addition, a second round of pappus hairs was observed (B, D). SEM analysis of the transgenic petals (D) revealed that at the base of the extra pappus there are round-shaped cells similar to cells observed in the wild type (C) in a ring between the ovary and pappus hairs. Scale bars, 100 μm.

In chrysanthemum, yeast three-hybrid analyses have shown that the two eu*AP3*-type proteins CDM115 and CDM19, corresponding to GDEF2 and GDEF3, respectively, act differentially in ternary complex formation (Shchennikova *et al.*, 2004). They both form heterodimers with the *PI*/*GLO*-type protein CDM86, but only the CDM86–CDM115 heterodimer forms a ternary complex with *AP1*/*FUL*-type proteins and with a class C protein, whereas the CDM86–CDM19 heterodimer does not. To investigate if this apparent diversification in functions is conserved, a yeast three-hybrid experiment was performed for the gerbera GDEF2 and GDEF3 proteins. It was found, however, that gerbera GGLO1–GDEF2 and GGLO1–GDEF3 heterodimers were both capable of forming ternary complexes with the *AP1*/*FUL*-type and the C class proteins (Supplementary Fig. S7 at *JXB* online).

Discussion

Asteraceae-specific B gene duplications and conservation of function

The transgenic phenotypes show that the *PI*/*GLO* and eu*AP3* lineage genes *GGLO1* and *GDEF2* encode the classical B-function in gerbera. Reduction in the expression of *GGLO1* and *GDEF2* results in homeotic conversion of stamens towards carpels and of petals towards pappus bristles (modified sepals). Moreover, the expression of *GGLO1*, as well as both the eu*AP3* lineage genes *GDEF2* and *GDEF3*, is ubiquitous throughout the development of petals and stamens.

The presence of a paralogous pair of eu*AP3* lineage genes is shared by gerbera, chrysanthemum, and sunflower. This reflects a common origin, possibly from the genome-wide duplication at the base of the Asteraceae lineage (Barker *et al.*, 2008). It is unlikely that duplicated genes would have avoided non-functionalization or loss in all three lines during the evolutionary history of Asteraceae [~40–49 million years ago (Mya); Kim *et al.*, 2005; Barker *et al.*, 2008] if they were completely functionally redundant. The average half-life for redundant gene duplicates is only 4 million years (Lynch and Conery, 2003). This implies that the pair of euAP3 lineage genes has probably undergone functional diversification (e.g. sub- or neofunctionalization). However, the *GDEF2* and *GDEF3* sequences and expression patterns are very similar and their protein interaction capacities seem not to differ, suggesting that they are at least partially redundant. In support of functional diversification, however, in transgenic lines that express *GGLO1* ectopically, it was observed that the expression of *GDEF2* and *GDEF3* is induced differentially.

Sunflower shows the presence of a second *PI*/*GLO*-like gene (Dezar *et al.*, 2003; Shulga *et al.*, 2008). An orthologue has not been reported in chrysanthemum and, despite extensive efforts taken to find a second *PI*/*GLO*-like gene, gerbera seems to have only *GGLO1*. An analysis of the gerbera floral transcriptome using EST sequencing (Laitinen *et al.*, 2005, and unpublished sequences obtained by 454 pyrosequencing) has yielded >100 tags similar to *GGLO1*, but none for a second *GGLO* gene. The lack of a second *GLO*-like gene in gerbera and chrysanthemum suggests that the sunflower branch of Asteraceae has had a recent *PI*/*GLO* duplication.

B gene expression and autoregulation

The pattern of gerbera B class gene expression in flowers is comparable with that of *Antirrhinum*, petunia, and tobacco, where *PI*/*GLO*-like genes show strictly restricted expression and eu*AP3* genes a more widespread expression in all four floral organs (Sommer *et al.*, 1990; Davies *et al.*, 1996; Hileman and Irish, 2009). This pattern deviates from that of *Arabidopsis*, where, in addition to petals and stamens, *PI* transcripts are detectable in the inner (destined to carpels) and *AP3* in the outer (destined to sepals) parts of the flower primordia early in development (Weigel and Meyerowitz, 1993; Goto and Meyerowitz, 1994). This initial expression

Downloaded from https://academic.oup.com/jxb/article/61/1/75/566697 by guest on 24 April 2024

pattern is evoked independently, but at later developmental stages the expression is regulated through an auto- and cross-regulatory loop, in which the formation of a heterodimer between *PI*/*GLO* and eu*AP3* members is required to maintain expression of these genes specifically in petals and stamens (Schwarz-Sommer *et al.*, 1992; Halfter *et al.*, 1994; Jack *et al.*, 1994).

In gerbera, it was found that ectopic expression of *GGLO1* leads to an up-regulation of the expression of *GDEF1*, *GDEF2*, and *GDEF3* (albeit to different levels; see Supplementary Fig. S5 at *JXB* online). Remarkably, however, in the transgenic line with reduced *GGLO1* expression, homeotic alterations were detected but *GDEF2* (as well as *GDEF1* or *GDEF3*) transcription was not diminished. Apparently, even though *GGLO1* is capable of regulating *GDEF* expression, *GGLO1* expression is not necessary for the maintenance of *GDEF* expression. Alternatively, a very low level of *GGLO1* is sufficient for autoregulation. The vegetative expression of *GDEF2* in tissues where *GGLO1* is not expressed further supports the presence of a regulatory mechanism independent from autoregulation.

Transgenic phenotypes suggest a sepaloid origin for the gerbera ovary wall

The homeotic conversion of petals caused by reduced B class gene expression has characteristics unique to gerbera. The abaxial epidermis of basal petal tubes takes on the identity of ovary wall (and not whorl one, which in gerbera is occupied by pappus bristles). The resemblance of petal tubes to ovary wall is especially strong in transgenic lines with reduced *GDEF2* expression. Gerbera flowers are epigynous, as they have an inferior ovary located below the floral organs. There are two major morphological interpretations about the origin of the inferior ovary wall; it may be part of the floral axis (and thus be receptacular) or may result from fusion of floral organs with the ovary (and therefore be appendicular) (Gustafsson and Albert, 1999). The present transgenic loss-of-B-function phenotypes showing ovary wall-like tissues in petals suggest the latter alternative, i.e. that the ovary wall might have sepaloid origin, having evolved through fusion of the perianth organs with the ovary.

GDEF1 *may act redundantly in stamen development*

Expression of the *TM6* lineage gene *GDEF1* differs from that of the other gerbera B class genes in several aspects. Instead, it shows similarities with the pattern of *PhTM6* expression in petunia (Vandenbussche *et al.*, 2004). Both genes are expressed in all four floral organs and most strongly in stamens and carpels. In petals, their expression becomes similarly weaker at later stages of development. However, there are differences in the initial onset of the expression. In gerbera, *GDEF1* transcripts are not detected in the region of emerging petal primordia, whereas in petunia and also in tomato (de Martino *et al.*, 2006), the expression in petals begins already at the primordium stage. The pattern of *GDEF1* expression in petals is spatially restricted to lateral edges. Similarly, the tomato *TM6* gene shows restricted expression in the lateral margins of petals (de Martino *et al.*, 2006). Adding to the complexity of the *GDEF1* expression pattern, stronger and more consistent expression of *GDEF1* was detected in disk flower petals than in ray flower petals.

Unlike other B class genes, *TM6*-like genes are prevalently expressed in carpels. Similarly to gerbera, petunia, and tomato, the grapevine *VvTM6* gene shows expression in carpels and ovules in addition to petals and stamens (Poupin *et al.*, 2007). Both in gerbera and in petunia (Vandenbussche *et al.*, 2004), *GDEF1* and *PhTM6* expression levels remain high in carpels until very late stages of flower development. Whether this expression in carpels is functionally relevant is currently unknown.

Despite the clearly distinct expression pattern, transgenic plants in which *GDEF1* expression was suppressed did not reveal any unique phenotypes but only very mild phenotypic changes. Similarly, in petunia, down-regulation of *PhTM6* expression alone did not cause any phenotypic changes, and only analysis of the double mutant *phtm6 phdef* uncovered the role of *PhTM6* in stamens (Rijpkema *et al.*, 2006). Although there are no data from simultaneous down-regulation of *GDEF1* and *GDEF2* (or *GDEF3*), the present results indicate that *GDEF1* is not involved in determining petal identity. In addition to the lack of consistent expression of *GDEF1* in petals, further evidence comes from studying *GDEF1* expression in *GGLO1* overexpression lines, where the identity of pappus bristles has changed into petals. Although *GDEF1* is expressed in wild-type pappus bristles, in these homeotic petal-like organs *GDEF1* expression is clearly diminished, suggesting that homeotic conversion to petals may in fact require reduction in *GDEF1* expression.

The mildness of stamen phenotype due to reduced *GDEF1* expression indicates a redundant role for this gene. Suppressed *GDEF1* expression caused earlier senescence and diminished release of pollen in disk flower stamens. *GDEF1* expression persists longer in wild-type stamens than that of *GDEF2* or *GDEF3*, suggesting that *GDEF1* may have an independent regulatory role in late stages of stamen development. However, *GDEF1* cannot compensate for the lack of *GDEF2* expression in stamens, since similar and stronger changes are detected in the *GDEF2* transgenic lines. To define the distribution of regulatory roles between the three *AP3*-type genes in gerbera, production of *GDEF3* down-regulation lines is required. Furthermore, transgenic lines in which two or all of the three genes are down-regulated simultaneously would be informative concerning the overlapping roles that the present work suggests.

Conclusions

The present analysis of B-function genes in gerbera has uncovered many commonalities with the function of orthologues in other systems, particularly other asterids such as Solanaceae. Importantly, gerbera represents an entirely

Downloaded from https://academic.oup.com/jxb/article/61/1/75/566697 by guest on 24 April 2024

distinct lineage of the asterids that diverged from the lineage containing Solanaceae >100 Mya (Wikström *et al.*, 2001). As such, the general details of eu*AP3*/*TM6*-like gene function described here can be parsimoniously interpreted as the basal state among the asterids. Although drastic modification has occurred at least once, in *Antirrhinum* (Lamiales), which has lost its *TM6* copy (as has *Arabidopsis* independently), it can be inferred that even though B-function expression details can be flexible in asterids (Hileman and Irish, 2009), their function has persisted across vast expanses of time with only few modifications.

Supplementary data

Supplementary data are available at *JXB* online and comprise the following figures and tables.

Figure S1. Amino acid alignment for a selected set of eu*AP3*-clade MADS-box genes.

Figure S2. Expression of the B class genes in gerbera disc flowers during development of petals, stamens, and carpels.

Figure S3. Expression of the B class genes in young inflorescences of the transgenic gerbera lines.

Figure S4. SEM analysis of the transgenic gerbera line (Tr22) with reduced *GDEF2* expression.

Figure S5. Expression of the gerbera B class genes in the transgenic *GGLO1* overexpression line.

Figure S6. Yeast two-hybrid analysis of the gerbera B class MADS domain proteins.

Figure S7. Yeast three-hybrid analysis of the gerbera B class MADS domain proteins.

Table S1. The B class genes used in the phylogenetic analysis.

Table S2. Primers used for Gateway conversion of the gerbera B class MADS-box genes.

Acknowledgements

We thank the Electron Microscopy Unit of the Institute of Biotechnology, University of Helsinki, for providing laboratory facilities; Eija Takala and Anu Rokkanen for excellent technical assistance; Sanna Peltola for taking care of the plants in the greenhouse; and Anneke Rijpkema for critical reading and helping to improve the manuscript. The Centre of Excellence program of the Academy of Finland supported this work. SKB was supported by the Viikki Graduate School in Biosciences.

References

Angiosperm Phylogeny Group II. 2003. An update of the angiosperm phylogeny group classification for the orders and families of flowering plants: APG II. *Botanical Journal of the Linnean Society* **141,** 399–436.

Barker MS, Kane NC, Matvienko M, Kozik A, Michelmore RW, Knapp SJ, Rieseberg LH. 2008. Multiple paleopolyploidizations during the evolution of the Compositae reveal parallel patterns of duplicate gene retention after millions of years. *Molecular Biology and Evolution* **25,** 2445–2455.

Broholm SK, Tähtiharju S, Laitinen RA, Albert VA, Teeri TH, Elomaa P. 2008. A TCP domain transcription factor controls flower type specification along the radial axis of the *Gerbera* (Asteraceae) inflorescence. *Proceedings of the National Academy of Sciences, USA* **105,** 9117–9122.

Davies B, Di Rosa A, Eneva T, Saedler H, Sommer H. 1996. Alteration of tobacco floral organ identity by expression of combinations of Antirrhinum MADS-box genes. *The Plant Journal* **10,** 663–677.

de Martino G, Pan I, Emmanuel E, Levy A, Irish VF. 2006. Functional analyses of two tomato *APETALA3* genes demonstrate diversification in their roles in regulating floral development. *The Plant Cell* **18,** 1833–1845.

Dezar CA, Tioni MF, Gonzalez DH, Chan RL. 2003. Identification of three MADS-box genes expressed in sunflower capitulum. *Journal of Experimental Botany* **54,** 1637–1639.

Elomaa P, Teeri TH. 2001. Transgenic *Gerbera*. In: Bajaj YPS, ed. *Biotechnology in agriculture and forestry* 48. Berlin: Springer-Verlag, 139–154.

Elomaa P, Uimari A, Mehto M, Albert VA, Laitinen RAE, Teeri TH. 2003. Activation of anthocyanin biosynthesis in *Gerbera hybrida* (Asteraceae) suggests conserved protein–protein and protein–promoter interactions between the anciently diverged monocots and eudicots. *Plant Physiology* **133,** 1831–1842.

Felsenstein J. 1985. Confidence-limits on phylogenies—an approach using the bootstrap. *Evolution* **39,** 783–791.

Gascuel O. 1997. BIONJ: an improved version of the NJ algorithm based on a simple model of sequence data. *Molecular Biology and Evolution* **14,** 685–695.

Goto K, Meyerowitz EM. 1994. Function and regulation of the *Arabidopsis* floral homeotic gene *PISTILLATA*. *Genes and Development* **8,** 1548–1560.

Guindon S, Gascuel O. 2003. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Systematic Biology* **52,** 696–704.

Guindon S, Lethiec F, Duroux P, Gascuel O. 2005. PHYML online—a web server for fast maximum likelihood-based phylogenetic inference. *Nucleic Acids Research* **33,** W557–W559.

Gustafsson MHG, Albert VA. 1999. Inferior ovaries and angiosperm diversification. In: Hollingsworth PM, Bateman RM, Gornall RJ, eds. *Molecular systematics and plant evolution,* The Systematics Association Special Volume Series edn. New York: Taylor and Francis, 403–431.

Halfter U, Ali N, Stockhaus J, Ren L, Chua NH. 1994. Ectopic expression of a single homeotic gene, the petunia gene green petal, is sufficient to convert sepals to petaloid organs. *EMBO Journal* **13,** 1443–1449.

Hasegawa M, Kishino H, Yano T. 1985. Dating of the human–ape splitting by a molecular clock of mitochondrial DNA. *Journal of Molecular Evolution* **22,** 160–174.

Helariutta Y, Elomaa P, Kotilainen M, Seppänen P, Teeri TH. 1993. Cloning of cDNA coding for dihydroflavonol-4-reductase (DFR) and characterization of *dfr* expression in the corollas of *Gerbera hybrida* var. Regina (Compositae). *Plant Molecular Biology* **22,** 183–193.

Downloaded from https://academic.oup.com/jxb/article/61/1/75/566697 by guest on 24 April 2024

Hernandez-Hernandez T, Martinez-Castilla LP, Alvarez-Buylla ER. 2007. Functional diversification of B MADS-box homeotic regulators of flower development: adaptive evolution in protein–protein interaction domains after major gene duplication events. *Molecular Biology and Evolution* **24,** 465–481.

Hileman LC, Irish VF. 2009. More is better: the uses of developmental genetic data to reconstruct perianth evolution. *American Journal of Botany* **96,** 83–95.

Jack T, Fox GL, Meyerowitz EM. 1994. Arabidopsis homeotic gene *APETALA3* ectopic expression: transcriptional and posttranscriptional regulation determine floral organ identity. *Cell* **76,** 703–716.

James P, Halladay J, Craig EA. 1996. Genomic libraries and a host strain designed for highly efficient two-hybrid selection in yeast. *Genetics* **144,** 1425–1436.

Kim KJ, Choi KS, Jansen RK. 2005. Two chloroplast DNA inversions originated simultaneously during the early evolution of the sunflower family (Asteraceae). *Molecular Biology and Evolution* **22,** 1783–1792.

Kim S, Yoo M, Albert VA, Farris JS, Soltis PS, Soltis DE. 2004. Phylogeny and diversification of B-function MADS-box genes in angiosperms: evolutionary and functional implications of a 260-million-year-old duplication. *American Journal of Botany* **91,** 2102–2118.

Kramer EM, Dorit RL, Irish VF. 1998. Molecular evolution of genes controlling petal and stamen development: duplication and divergence within the *APETALA3* and *PISTILLATA* MADS-box gene lineages. *Genetics* **149,** 765–783.

Kramer EM, Irish VF. 2000. Evolution of the petal and stamen developmental programs: evidence from comparative studies of the lower eudicots and basal angiosperms. *International Journal of Plant Sciences* **161,** S29–S40.

Kramer EM, Jaramillo MA, Di Stilio VS. 2004. Patterns of gene duplication and functional evolution during the diversification of the *AGAMOUS* subfamily of MADS box genes in angiosperms. *Genetics* **166,** 1011–1023.

Kramer EM, Su HJ, Wu CC, Hu JM. 2006. A simplified explanation for the frameshift mutation that created a novel C-terminal motif in the *APETALA3* gene lineage. *BMC Evolutionary Biology* **6,** 30.

Laitinen RA, Ainasoja M, Broholm SK, Teeri TH, Elomaa P. 2008. Identification of target genes for a MYB-type anthocyanin regulator in *Gerbera hybrida*. *Journal of Experimental Botany* **59,** 3691–3703.

Laitinen RAE, Immanen J, Auvinen P, *et al*. 2005. Analysis of the floral transcriptome uncovers new regulators of organ determination and gene families related to flower organ differentiation in *Gerbera hybrida* (Asteraceae). *Genome Research* **15,** 475–486.

Lamb RS, Irish VF. 2003. Functional divergence within the *APETALA3/PISTILLATA* floral homeotic gene lineages. *Proceedings of the National Academy of Sciences, USA* **100,** 6558–6563.

Lynch M, Conery JS. 2003. The evolutionary demography of duplicate genes. *Journal of Structural and Functional Genomics* **3,** 35–44.

Piwarzyk E, Yang Y, Jack T. 2007. Conserved C-terminal motifs of the arabidopsis proteins APETALA3 and PISTILLATA are dispensable for floral organ identity function. *Plant Physiology* **145,** 1495–1505.

Poupin MJ, Federici F, Medina C, Matus JT, Timmermann T, Arce-Johnson P. 2007. Isolation of the three grape sub-lineages of B-class MADS-box *TM6*, *PISTILLATA* and *APETALA3* genes which are differentially expressed during flower and fruit development. *Gene* **404,** 10–24.

Rijpkema AS, Royaert S, Zethof J, van der Weerden G, Gerats T, Vandenbussche M. 2006. Analysis of the *Petunia TM6* MADS box gene reveals functional divergence within the *DEF/AP3* lineage. *The Plant Cell* **18,** 1819–1832.

Schwarz-Sommer Z, Hue I, Huijser P, Flor PJ, Hansen R, Tetens F, Lönnig WE, Saedler H, Sommer H. 1992. Characterization of the *Antirrhinum* floral homeotic MADS-box gene *deficiens*: evidence for DNA binding and autoregulation of its persistent expression throughout flower development. *EMBO Journal* **11,** 251–263.

Shchennikova AV, Shulga OA, Immink R, Skryabin KG, Angenent GC. 2004. Identification and characterization of four chrysanthemum MADS-box genes, belonging to the *APETALA1/ FRUITFULL* and *SEPALLATA3* subfamilies. *Plant Physiology* **134,** 1632–1641.

Shulga OA, Shchennikova AV, Angenent GC, Skryabin KG. 2008. *MADS*-box genes controlling inflorescence morphogenesis in sunflower. *Russian Journal of Developmental Biology* **39,** 2–5.

Sommer H, Beltran JP, Huijser P, Pape H, Lonnig WE, Saedler H, Schwarz-Sommer Z. 1990. *Deficiens*, a homeotic gene involved in the control of flower morphogenesis in *Antirrhinum majus*: The protein shows homology to transcription factors. *EMBO Journal* **9,** 605–613.

Su K, Zhao S, Shan H, Kong H, Lu W, Theissen G, Chen Z, Meng Z. 2008. The MIK region rather than the C-terminal domain of AP3-like class B floral homeotic proteins determines functional specificity in the development and evolution of petals. *New Phytology* **178,** 544–558.

Tröbner W, Ramirez L, Motte P, Hue I, Huijser P, Lonnig WE, Saedler H, Sommer H, Schwarz-Sommer Z. 1992. *GLOBOSA*: a homeotic gene which interacts with *DEFICIENS* in the control of *Antirrhinum* floral organogenesis. *EMBO Journal* **11,** 4693–4704.

Uimari A, Kotilainen M, Elomaa P, Yu D, Albert VA, Teeri TH. 2004. Integration of reproductive meristem fates by a *SEPALLATA*-like MADS-box gene. *Proceedings of the National Academy of Sciences, USA* **101,** 15817–15822.

Vandenbussche M, Theissen G, Van de Peer Y, Gerats T. 2003. Structural diversification and neo-functionalization during floral MADS-box gene evolution by C-terminal frameshift mutations. *Nucleic Acids Research* **31,** 4401–4409.

Vandenbussche M, Zethof J, Royaert S, Weterings K, Gerats T. 2004. The duplicated B-class heterodimer model: whorl-specific effects and complex genetic interactions in *Petunia hybrida* flower development. *The Plant Cell* **16,** 741–754.

Weigel D, Meyerowitz EM. 1993. Activation of floral homeotic genes in arabidopsis. *Science* **261,** 1723–1726.

Wikström N, Savolainen V, Chase MW. 2001. Evolution of the angiosperms: calibrating the family tree. *Proceedings of the Royal Society B: Biological Sciences* **268,** 2211–2220.

Yu D, Kotilainen M, Pöllänen E, Mehto M, Elomaa P, Helariutta Y, Albert VA, Teeri TH. 1999. Organ identity genes and modified patterns of flower development in *Gerbera hybrida* (Asteraceae). *The Plant Journal* **17,** 51–62.

Downloaded from https://academic.oup.com/jxb/article/61/1/75/566697 by guest on 24 April 2024