The role of MADS and TCP transcription factors in *Gerbera hybrida* flower development

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List of original publications

This thesis is based on the following publications and a manuscript. The publications are referred to in the text by their roman numerals. The publications have been reprinted with a kind permission from the publishers.

I
Roosa A. E. Laitinen, **Suvi Broholm**, Victor A. Albert, Teemu H. Teeri and Paula Elomaa (2006). Patterns of MADS-box gene expression mark flower-type development in *Gerbera hybrida* (Asteraceae). BMC Plant Biology 6:11.

II
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III
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Author's contribution

I
SB was responsible for carrying out the histological and SEM analyses. The publication was part of RAEL's thesis and she did the microarray experiments, analyzed the data and wrote the manuscript together with VAA and PE.

II
SB was responsible for majority of the experimental work, including sequence alignment, expression analyses, transformation of gerbera and analysis of the transgenic phenotypes. ST, PE and RAEL isolated the genes and VAA constructed the phylogenetic tree. SB wrote the manuscript together with VAA, THT, and PE.

III
SB has had a major contribution to the following tasks: cloning of *GDEF3*, sequence analyses and alignments, expression analyses, transformation of gerbera (*GDEF1*-lines) and analysis of the transgenic phenotypes. The *GGLO1*- and *GDEF2*-lines have been analyzed together with EP and MK. SR and ST contributed to the yeast two-hybrid analysis. VAA constructed the phylogenetic tree. SB wrote the manuscript together with VAA, PE, and THT.

Abbreviations

bHLH	basic-helix-loop-helix
cDNA	complementary DNA
EST	Expressed Sequence Tag
evo-devo	evolutionary developmental genetics
FDR	False Discovery Rate
FM	Floral meristem
IM	Inflorescence meristen
miRNA	microRNA
MYA	Million Years Ago
qPCR	quantitative real time polymerase chain reaction
RNAi	RNA interference
SAM	shoot apical meristem
SEM	scanning electron microscopy

Abstract

Angiosperms represent a huge diversity in floral structures. Thus, they provide an attractive target for comparative developmental genetics studies. Research on flower development has focused on few main model plants, and studies on these species have revealed the importance of transcription factors, such as MADS-box and TCP genes, for regulating the floral form. The MADS-box genes determine floral organ identities, whereas the TCP genes are known to regulate flower shape and the number of floral organs. In this study, I have concentrated on these two gene families and their role in regulating flower development in *Gerbera hybrida*, a species belonging to the large sunflower family (Asteraceae).

The *Gerbera* inflorescence is comprised of hundreds of tightly clustered flowers that differ in their size, shape and function according to their position in the inflorescence. The presence of distinct flower types tells *Gerbera* apart from the common model species that bear only single kinds of flowers in their inflorescences. The marginally located ray flowers have large bilaterally symmetrical petals and non-functional stamens. The centrally located disc flowers are smaller, have less pronounced bilateral symmetry and carry functional stamens. Early stages of flower development were studied in *Gerbera* to understand the differentiation of flower types better. After morphological analysis, we compared gene expression between ray and disc flowers to reveal transcriptional differences in flower types. Interestingly, MADS-box genes showed differential expression, suggesting that they might take part in defining flower types by forming flower-type-specific regulatory complexes.

Functional analysis of a *CYCLOIDEA*-like TCP gene *GhCYC2* provided evidence that TCP transcription factors are involved in flower type differentiation in *Gerbera*. The expression of *GhCYC2* is ray-flower-specific at early stages of development and activated only later in disc flowers. Overexpression of *GhCYC2* in transgenic *Gerbera*-lines causes disc flowers to obtain ray-flower-like characters, such as elongated petals and disrupted stamen development. The expression pattern and transgenic phenotypes further suggest that *GhCYC2* may shape ray flowers by promoting organ fusion. Cooperation of *GhCYC2* with other *Gerbera CYC*-like TCP genes is most likely needed for proper flower type specification, and by this means for shaping the elaborate inflorescence structure.

Gerbera flower development was also approached by characterizing B class MADS-box genes, which in the main model plants are known regulators of petal and stamen identity. The four *Gerbera* B class genes were phylogenetically grouped into three clades; *GGLO1* into the *PI/GLO* clade, *GDEF2* and *GDEF3* into the eu*AP3* clade and *GDEF1* into the *TM6* clade. Putative orthologs for *GDEF2* and *GDEF3* were identified in other Asteraceae species, which suggests that they appeared through an Asteraceae-specific duplication. Functional analyses indicated that *GGLO1* and *GDEF2* perform conventional B-function as they determine petal and stamen identities. Our studies on *GDEF1* represent the first functional analysis of a *TM6*-like gene outside the Solanaceae lineage and provide further evidence for the role of *TM6* clade members in specifying stamen development. Overall, the *Gerbera* B class genes showed both commonalities and diversifications with the conventional B-function described in the main model plants.

1 INTRODUCTION

1.1 Evolutionary developmental genetics of flowers

Explaining the mechanisms behind the enormous diversity of plant and animal body plans is one of the greatest challenges in biology. One way to approach the mysteries of evolution is evolutionary developmental genetics ("evo-devo"). Evo-devo aims at explaining how morphological variations arise and thereby provide material for evolution. The basic idea behind evo-devo is that there is interdependence between evolution and development. This was realized as early as the 19th century, but it was not until the 1980s that evo-devo emerged as a distinct field of research (Theissen and Saedler, 1995). The power of evo-devo comes from its ability to fill the gap between the interplay of mutations and selection, which are major concepts in the theory of evolution. Mutations provide new genes but selection does not act on genes, it acts on phenotypes (Arthur, 2002). Development of a multicellular organism from a single cell (zygote) is under genetic control and thus mutations in the genes driving this process may alter the phenotype so that the evolution can proceed through selection. Thus, changes in the major developmental control genes are especially important in generating morphological novelty.

Majority of the genes guiding developmental processes are members of the multigene families that encode transcription factors, which are proteins that bind to the DNA by recognizing specific sequences (*cis*-elements) and thereby regulate expression of their target genes. Understanding the phylogeny of these genes will help to understand the evolution of organisms. Moreover, comparing the spatiotemporal patterns of expression and function of these genes between species showing diverged morphologies will direct our understanding on the mechanisms of evolution.

Flowering plants (angiosperms) are the dominant form of plant life on land. Thus, the emergence of hermaphroditic reproductive axes with closed carpel and double fertilization has been a key innovation in plant evolution (Theissen and Melzer, 2007). Key innovations are advantageous traits that become fixed and promote evolutionary radiations. The importance of flowers and inflorescences for the evolutionary success of angiosperms and their extensive morphological variations makes them attractive targets for studying the evolution of developmental mechanisms. Flowers are morphologically the most complicated parts of plants. Instead of being single organs, such as stems, leaves, and roots, flowers are composite structures comprising a number of organs. Flowers are often clustered to form a more elaborate inflorescence structure, thus raising the complexity to a further level. The key regulators of floral development belong to relatively few gene families. In this thesis, I have concentrated on two of these families, the MADS and TCP transcription factors, which will be described in more detail.

1.2 MADS-box genes in flower development

1.2.1 Floral organ identities and the ABCDE-model

Despite the diversity of forms, most flowers share a common basic architecture, in which four floral organs are organized in concentric whorls. There are sepals in whorl 1, petals in whorl 2, stamens in whorl 3 and carpels in whorl 4. The key developmental genes that regulate the identity of floral organs belong to the MADS-box gene family. The role of MADS-box genes in flower development was revealed during late 1980s and early 1990s, and was based on forward genetic studies on homeotically altered mutant flowers. Studies with model species, mainly *Arabidopsis thaliana* and *Antirrhinum majus*, yielded the classic ABC model that describes the genetic basis for floral organ determination (Coen and Meyerowitz, 1991). According to this model, floral organs adopt their identities through combinatorial interaction of three classes of floral homeotic functions, with class A specifying sepals, A and B petals, B and C stamens, and C alone carpels. Mutual repression between A and C functions and the regulation of floral meristem determinacy by C function were also included in the model based on mutant analyses (Bowman *et al.*, 1991; Meyerowitz *et al.*, 1991). Further functions, D and E, have been added to the model later, thus receiving an extended ABCDE model. The D class genes were identified by studies on another model species, *Petunia hybrida*, and were shown to be necessary for determining ovule identity (Angenent *et al.*, 1995; Colombo *et al.*, 1995). Moreover, the E class genes were demonstrated to act together with the ABC genes in defining the identity of sepals, petals, stamens and carpels (Pelaz *et al.*, 2000; Theissen and Saedler, 2001; Ditta *et al.*, 2004). In fact, coexpression of an E class gene with the ABC genes is sufficient to turn leaves into floral organs (Honma and Goto, 2001)

The MADS-box genes behind the ABCDE functions have been isolated and characterized from several species. In *Arabidopsis*, A function is determined by *APETALA1* (*AP1*) and *APETALA2* (*AP2*), of which *AP2* is the only ABCDE gene that does not belong to the MADS family (Mandel *et al.*, 1992; Jofuku *et al.*, 1994). The B-function is determined by two related genes, *APETALA3* (*AP3*) and *PISTILLATA* (*PI*), whereas the C-function is defined by a single gene *AGAMOUS* (*AG*). The late discovery of the E-function was due to a high level of genetic redundancy, as there are four genes behind this function, namely *SEPALLATA1*, *2*, *3*, and *4* (*SEP1-4*, formerly *AGL2,4,9,3*). Single mutants of the *SEP* genes cause none or only very mild phenotypes but triple *sep1/2/3* mutant flowers solely consist of sepals (Pelaz *et al.*, 2000). The quadruple *sep1/2/3/4* mutant displays indeterminate flowers composed of leaf-like organs (Ditta *et al.*, 2004). Orthologs of the B, C, and E genes from selected model plants, both eudicots and monocots, have been collected in Table 1.

The classic ABC model has been very useful for producing testable hypotheses for comparative studies, thus accelerating the progress of floral evo-devo studies. Advances in the field have revealed the complexity of genetic interactions, as shown by the addition of D- and E-functions. Comparative studies have revealed both conservation and diversification of mechanisms between species. As an example, the A function, as defined in the original ABC-model, has not been described in any other species than *Arabidopsis* (and is thus excluded from Table 1). The *Arabidopsis* A function genes *AP1* and *AP2* have three subfunctions. In addition to determining sepal and petal identities, they control spatial restriction of the C genes to the inner floral whorls and also exert a central role in the establishment of floral meristem identity (Mandel *et al.*, 1992; Jofuku *et al.*, 1994). The *AP1* orthologs in other species seem to share only the floral meristem identity function (Huijser *et al.*, 1992; Taylor *et al.*, 2002). The *AP2* orthologs *LIP1* and *LIP2* in *Antirrhinum* take part in sepal and petal development, but not in repressing the C genes (Keck *et al.*, 2003). Mechanisms that pattern C gene expression were originally confined to the A genes, but have since been shown to be diverse. For example, *blind* and *fistulata* mutants have similar homeotic phenotypes with the conversion of petals into stamens in both *Petunia* and *Antirrhinum*. Cloning the genes behind these mutants revealed the role of microRNAs (miRNA) in controlling C gene expression in these species but not in *Arabidopsis* (Cartolano *et al.*, 2007).

Table 1 *The B, C, and E class MADS-box genes in selected plant species. The classifications are based on either functional orthology or sequence homology.* Oryza sativa *(rice) represents a monocot, whereas all the other species are core eudicots. Within the B class, members of the eu*AP3*-clade (paleo*AP3*-clade in rice) are marked with A, the* TM6*-clade members with T and the* PI*-clade members with P.*

Species	B class	C class	E class	Reference
Arabidopsis thaliana	*AP3*$_A$, *PI*$_P$	*AG*	*SEP1 - 4*	Jack *et al.*, 1992; Goto and Meyerowitz, 1994; Yanofsky *et al.*, 1990; Pelaz *et al.*, 2000; Ditta *et al.*, 2004
Antirrhinum majus	*DEF*$_A$, *GLO*$_P$	*PLE, FAR*	*DEFH49, DEFH72, DEFH200*	Sommer *et al.*, 1990; Tröbner *et al.*, 1992; Bradley *et al.*, 1993; Davies *et al.*, 1996a, 1999
Petunia hybrida	*PhDEF*$_A$, *PhTM6*$_T$, *PhGLO1*$_P$, *PhGLO2*$_P$	*PMADS3, FBP6*	*FBP2, FBP5, pMADS12, FBP4, FBP9, FBP23*	van der Krol *et al.*, 1993; Kramer and Irish., 2000; Angenent *et al.*, 1992, 1993; Tsuchimoto *et al.*, 1993; Immink *et al.*, 2002; Ferrario *et al.*, 2003
Gerbera hybrida	*GDEF2*$_A$, *GDEF3*$_A$, *GDEF1*$_T$, *GGLO1*$_P$	*GAGA1, GAGA2*	*GRCD1 - 5*	Yu *et al.*, 1999; Broholm *et al.*, manuscript (III); Kotilainen *et al.*, 2000; Uimari *et al.*, 2004; Laitinen *et al.*, 2005
Oryza sativa	*SPW1/OsMADS16*$_A$, *OsMADS2* $_P$, *OsMADS4*$_P$	*OsMADS3, OsMADS58*	*LHS1, OsMADS5 OsMADS7-8, OsMADS19, OsMADS34*	Moon *et al.*, 1999; Chung *et al.*, 1995; Kang *et al.*, 1997a, 1997b, 1998; Yamaguchi *et al.*, 2006; Malcomber and Kellog, 2004; Shinozuka *et al.*, 1999

1.2.2 The molecular basis of MADS-box gene functions

The defining characteristic of MADS transcription factors is the highly conserved MADS domain, which is approximately 58 amino acids long and possesses DNA-binding, dimerization and nuclear localization activities (Riechmann *et al.*, 1996a and 1996b). The abbreviation MADS is derived from the first four gene family members identified: MCM1 from yeast, the plant proteins AG and DEFA, and SRF from mammals (Schwarz-Sommer *et al.*, 1990). Most of the MADS proteins identified by analysing mutant phenotypes have a characteristic MIKC domain structure. In addition to the N-terminal MADS (M) domain, MIKC domain proteins are comprised of the less conserved intervening (I) domain and the keratin-like (K) domain, which also play roles in protein-protein interactions (Schwarz-Sommer *et al.*, 1992; Tröbner *et al.*, 1992; Riechmann *et al.*, 1996a). The C-terminal domain is again less conserved and contributes to higher order protein complexes between MADS proteins (Egea-Cortines *et al.*, 1999; Honma and Goto, 2001). In some MADS proteins, the C-terminus also contains a transcriptional activation domain (Cho *et al.*, 1999; Egea-Cortines *et al.*, 1999; de Folter *et al.*, 2005).

MADS domain proteins bind to DNA only as homo- or heterodimers, by recognizing specific palindromic DNA sequences with a core consensus sequence 5'-CC(A/T)$_6$GG-3', termed the CArG box (Schwarz-Sommer *et al.*, 1992; Tröbner *et al.*, 1992; Riechmann *et al.*, 1996a and 1996b). As to how MADS proteins recognize and exert differential effects on their specific target genes, though their DNA-binding sites are so similar, is still largely an unanswered question (Melzer *et al.*, 2006). The current explanations for defining target gene specificity range in the literature from differential DNA bending to protein dosage effects and cooperative binding with other protein partners.

The ability of the MADS domain proteins to form higher order complexes in plants has been proposed to be the molecular basis of the ABCDE functions, and thus led to a refined model of floral quartets (Honma and Goto, 2001; Theissen and Saedler, 2001). The floral quartet model postulates that the combinations of four MADS domain proteins form complexes that determine the identity of the floral organs. Sepals are defined by an AAEE quartet, petals by ABBE, stamens by BBCE, carpels by CCEE, and ovules by CDDE, referring to the ABCDE functions described earlier. The model explains well the role of the class E proteins by defining them as bridges for the formation of specific tetramers in all floral organs. Most of these protein interactions have been shown to occur in yeast (Davies *et al.*, 1996b; Egea-Cortines *et al.*, 1999; Favaro *et al.*, 2003; Honma and Goto, 2001; Shchennikova *et al.*, 2004; Leseberg *et al.*, 2008), but so far very few have been verified *in planta*. Especially the *in planta* stoichiometry is unknown and it may well be that the complexes are larger than tetramers and also contain non-MADS proteins, as indicated by several recent studies (Masiero *et al.*, 2002; Causier *et al.*, 2003; Sridhar *et al.*, 2006; He *et al.*, 2007).

1.2.3 MADS-box gene duplications and floral evolution

The MADS-box genes are present in plants, animals and fungi, thus they represent an ancient gene family. Phylogenetic reconstructions have identified two main lineages, namely type I and type II MADS-box genes. These were generated by a gene duplication that occurred in the common ancestor of the eukaryotic kingdom more than a billion years ago (Alvarez-Buylla *et al.*, 2000). The type I lineage members lack the K domain and have been subdivided into the Mα, Mβ and Mγ subfamilies (Parenicova *et al.*, 2003). The type II lineage consists of MIKC-type MADS domain proteins and can be further classified into MIKC^c-type and MIKC*-type, based on their intron–exon structures (Henschel *et al.*, 2002). Interestingly, all the MADS-box genes isolated by analysing phenotypic mutants have been MIKC^c-type genes, whereas only recently have a few other types of MADS-box genes been functionally characterized (Köhler *et al.*, 2003 and 2005; Portereiko *et al.*, 2006; Colombo *et al.*, 2008). The completely sequenced *Arabidopsis*, rice, and poplar genomes have totals of 107, 75, and 105 MADS-box genes, of which 39, 38, and 55 are MIKC^c-type genes, respectively (Parenicova *et al.*, 2003; Leseberg *et al.*, 2006; Arora *et al.*, 2007). The earliest land plant groups, mosses and lycopods, have one monophyletic group of MIKC^c-type genes and ferns have three monophyletic groups (Tanabe *et al.*, 2005). In contrast, 12 groups have been recognized in higher seed plants (Becker and Theissen, 2003). Thus, the extensive expansion of MIKC^c-type genes most probably occurred after the divergence of the seed plant lineage from ferns, though the possibility of extensive losses of genes in ferns and moss lineages and also the incomplete sampling of MADS-box genes in these species cannot be ruled out.

Formation of large gene families requires gene duplications and the preservation of duplicated gene copies through functional diversifications. Although a common fate for a duplicated copy is the accumulation of deleterious mutations and loss as a non-functionalized pseudogene, there are several ways for maintaining both copies. The original gene function may be divided into subfunctions that are shared between the duplicated gene copies (subfunctionalization), whereas in neofunctionalization one of the genes acquires a novel function (Force *et al.*, 1999). In the MADS-box gene family, preservation of duplicated genes with at least partially redundant functions, such as the *SEPALLATA* genes of *Arabidopsis* (Pelaz *et al.*, 2000), is also common. The advantages of maintaining redundant gene copies include an enhanced robustness to mutations (Wagner, 1999; Rijpkema *et al.*, 2007) and an improved fitness under varying natural conditions, that might be missed by mutant analysis under laboratory conditions (Briggs *et al.*, 2006).

Good examples of subfunctionalized genes are the rice C genes *OsMADS3* and *OsMADS58* (Table 1). The specification of C function in rice is divided so that *OsMADS3* is required for stamen identity, whereas *OsMADS58* is mainly involved in floral meristem determinacy and carpel morphogenesis (Yamaguchi *et al.*, 2006). Together these genes fulfill the functions that in *Arabidopsis* are accomplished by a single *AG* gene. Neofunctionalization is much rarer as it requires a mutation either in the regulatory or in coding region so that one of the duplicate genes acquires a new and useful function. The *Arabidopsis AP1/FUL*-like genes provide an example of partial redundancy and partial neofunctionalization. Triple mutation of *AP1*, *CAULIFLOWER* (*CAL*) and *FRUITFULL* (*FUL*) genes shows their partially overlapping (probably ancient) function in determining floral meristem identity (Ferrandiz *et al.*, 2000). Separate single mutant phenotypes indicate that they have also acquired unique functions in floral development. This is probably brought about by sequence evolution that has altered protein-protein interaction capabilities (Alvarez-Buylla *et al.*, 2006).

Large-scale genome duplications, such as polyploidization, are relatively common in plants and are mainly responsible for the large number of duplicated individual loci. It has been suggested that the *Arabidopsis* genome has undergone three whole-genome duplications (Bowers *et al.*, 2003). Interestingly, certain gene classes show differential retention when comparing large-scale and small-scale duplication events. In *Arabidopsis*, the retention of duplicated genes with regulatory functions, such as transcription factors, developmental and signal transduction genes has been more frequent after large-scale genome duplications than after small-scale duplications (Maere *et al.*, 2005). Large-scale gene duplication events have been associated with major leaps in the development and adaptive radiation of species. This highlights the importance of regulatory gene diversifications for such processes.

All major subfamilies of MADS-box genes that define the ABCDE functions have undergone a duplication event at the base of core eudicots. This putatively correlates with the second whole-genome duplication detected in the *Arabidopsis* genome (Kramer *et al.*, 1998; Becker and Theissen, 2003; Litt and Irish, 2003; Kramer *et al.*, 2004; De Bodt *et al.*, 2005; Zahn *et al.*, 2005a). Figure 1 combines the MADS-box gene duplications with a simplified angiosperm phylogeny, thereby relating the phylogeny of the MADS-box genes to angiosperm evolution. The duplication of *AP1/FUL*-lineage at the base of core eudicots gave rise to eu*AP1*- and eu*FUL*-lineages. All the monocot and basal angiosperm genes belong to a third lineage, named *FUL*-like lineage. The *FUL*-like lineage appears to be angiosperm specific as orthologs have not been identified in gymnosperms. The correlation of the origin of *AP1/FUL*-lineage with the origin of flowers suggests that these genes may have played a role in the evolution of this key angiosperm feature (Litt and Irish, 2003).

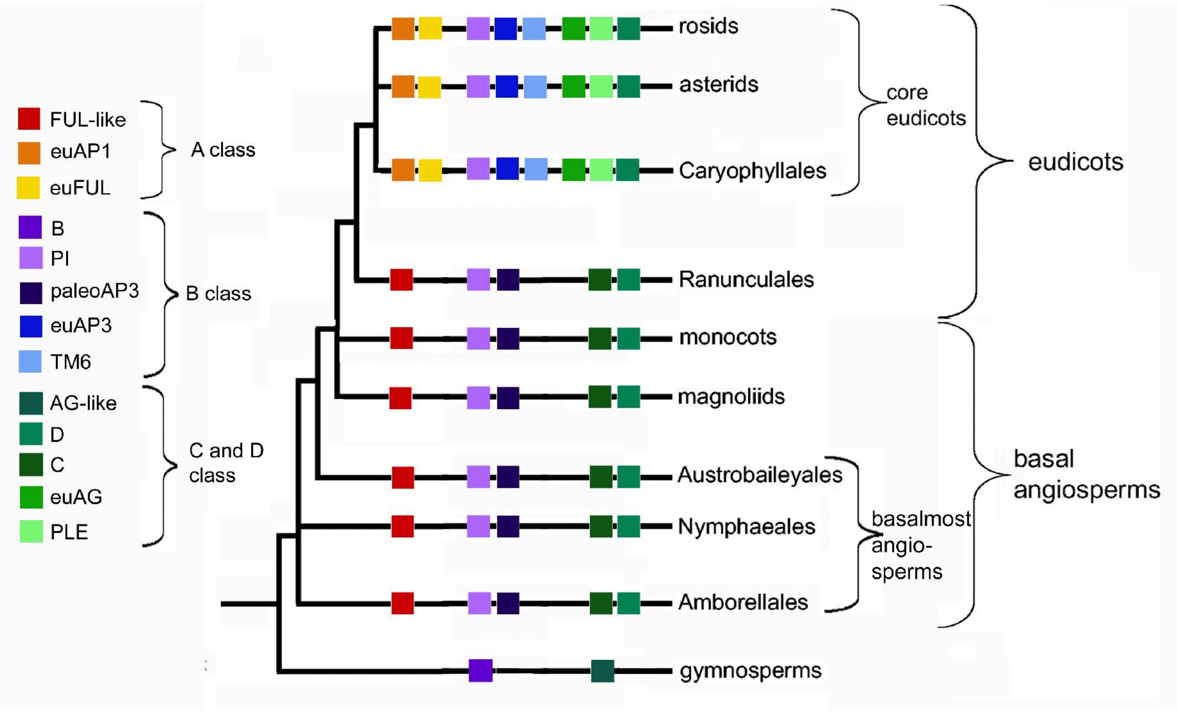

Figure 1 *The duplication history of MADS-box genes during angiosperm evolution. MIKC-type genes involved in determining floral organ identity are shown, except E class genes that have more complex duplication history (modified from Irish, 2003).*

The duplication history of B class genes is more complex. There has been a duplication event before the divergence of gymnosperm and angiosperm lineages that produced the closely related B and B_{sister} (B_s) genes (Becker *et al.*, 2002). The B_s genes are expressed in female reproductive organs (Becker *et al.*, 2002; Nesi *et al.*, 2002; Kaufmann *et al.*, 2005; de Folter *et al.*, 2006), in contrast to the B genes, which are predominantly expressed in male reproductive organs (Theissen and Becker, 2004; and refs in Table 1). Thus, it has been suggested that the last common ancestor of B and B_s genes was expressed in both male and female floral organs (Becker *et al.*, 2002). The B genes have undergone another major duplication event shortly after the divergence of extant gymnosperms and angiosperms that produced the *PI*- and *AP3*-like gene clades (Kim *et al.*, 2004; Hernandez-Hernandez *et al.*, 2007). The *AP3*-like genes have undergone a further duplication event at the base of core eudicots, resulting in eu*AP3*- and *TM6*-lineages (Kramer *et al.*, 1998). The C-terminal domain of the *TM6*-lineage genes, named the paleo*AP3* domain, is similar to the C-terminal domain of paleo*AP3*-type genes at the basal eudicots, monocots and basal angiosperms. The C-terminus of the core eudicot specific eu*AP3*-genes is derived from the paleo*AP3* domain via a frameshift mutation (Vandenbussche *et al.*, 2003; Kramer *et al.*, 2006).

Members of the *AG*-subfamily have been reported to have a conserved role in the development of reproductive organs in both gymnoperms and angiosperms, whereas ferns do not have *AG* orthologs (Becker *et al.*, 2000; Jager *et al.*, 2003). An ancient duplication event resulted in two major clades, termed C and D lineages (Kramer *et al.*, 2004). The D lineage includes the *Petunia* D class genes that were identified as regulators of ovule identity in dissociation from carpel identity (Colombo *et al.*, 1995). Although the D lineage members consistently show ovule-specific expression, the separation of C and D functions might not be universally applicable. The C and D lineage members have redundant roles in regulating ovule identity and thus the D function most likely represents a subfunctionalization of the C function (Kramer *et al.*, 2004). The C lineage has undergone a further major duplication. This duplication also appeared at the base of core eudicots and produced the eu*AG* and *PLE* lineages, which have maintained a high degree of conservation at both the level of sequence and function (Kramer *et al.*, 2004).

In addition to the ABCD genes described above, the E genes (*SEP*-clade) also have an interesting duplication history. The *SEP* genes have not been detected in gymnosperms but the first duplication event occurred in the lineage leading to extant angiosperms. Members of clades *AGL2/3/4* (*SEP1/4/2*) and *AGL9* (*SEP3*) have been isolated from the basalmost angiosperms such as Amborella, water lily, star anise and magnolia (Zahn *et al.*, 2005a). Several additional duplication events have occurred in monocots and dicots, leading to a complex duplication history (Zahn *et al.*, 2005a). The presence of the *SEP* homologs in all major lineages of angiosperms and their apparent absence in gymnosperms suggests that they have played a critical role in the origin of the flower (Zahn *et al.*, 2005a).

To conclude, the timings of the first duplication of *AP3/PI*-, *AG*-, and *SEP*-like genes overlap closely. This is thought to be a result of a whole-genome duplication event in the ancestor of extant angiosperms (De Bodt *et al.*, 2005; Zahn *et al.*, 2005a; Zahn *et al.*, 2005b). Moreover, a further duplication event in the *FUL*-, paleo*AP3*-, and C-lineages occurred coincidentally at the base of the core eudicot clade (Kramer and Hall, 2005). The former duplication event has been suggested to have contributed to the origin of the angiosperm flower, whereas the latter correlates with many significant changes in floral morphology that have influenced the radiation of core eudicots (De Bodt *et al.*, 2005; Kramer and Hall, 2005; Zahn *et al.*, 2005a, 2005b).

1.2.4 B class genes and evolution of petal identity pathways

The role of B class genes in specifying male reproductive organs has been shown to be largely conserved throughout the seed plants (Theissen and Becker, 2004). In contrast, the conservation of the petal identity pathway is less clear. The development of distinct petals and sepals (bipartite perianth) has been one of the key innovations behind the extensive radiation of flowering plants (Endress, 2001a). Two alternative schemes have been suggested for petal evolution; the bipartite perianth may have evolved several times independently or it might have been an ancestral trait, which has since been lost in multiple non-core eudicot lineages (Drea *et al.*, 2007). The assumption that petals have arisen multiple times is supported by the postulation that petals have been derived in some cases from stamens (andropetals) and in other cases from bracts (bracteopetals) (Hileman and Irish, 2009).

The duplication of the paleo*AP3*-clade into the eu*AP3*- and *TM6*-lineages at the base of core eudicots has been proposed to correlate with the origin of core eudicot petals (Kramer *et al.*, 1998; Kramer and Irish, 2000). The eu*AP3*-like gene underwent a C-terminal frameshift mutation and this was hypothesized to have led to a new function in petal development (Lamb and Irish, 2003; Vandenbussche *et al.*, 2003). However, there are contradictory results for the functional roles of the C-terminal paleo*AP3*- and eu*AP3*-domains (Lamb and Irish, 2003; Whipple *et al.*, 2004). The role of *TM6*-like genes in regulating only stamen but not petal identity (de Martino *et al.*, 2006; Rijpkema *et al.*, 2006) may not represent an ancestral functional restriction of paleo*AP3*-genes but a subfunctionalization event.

The importance of B class genes for petal development is highlighted by the 'sliding/shifting boundaries' model. This model demonstrates that an outward shift of B class gene expression can explain the petaloid nature of whorl one organs observed in several plant groups, which are often basal to the core eudicots (Albert *et al.*, 1998; Kramer *et al.*, 2003; Soltis *et al.*, 2006). In monocots, such as the lily family (species *Tulipa* and *Lilium*) and the orchid family, B gene homologs have been shown to be expressed in the petaloid outer floral whorl in addition to petals and stamens in whorls two and three (Kanno *et al.*, 2003; Tsai *et al.*, 2004; Xu *et al.*, 2006). Such findings are in accordance with the 'sliding/shifting boundaries' model. However, conflicting results showing that petaloid organs may also appear independent of B genes have been reported as well. Independence of early B gene expression has been shown for the petal-like perianth of a basal angiosperm *Aristolochia* (Jaramillo and Kramer, 2004). The petaloid sepals of a Ranunculales species *Aquilegia* (Kramer *et al.*, 2007) and a core eudicot *Impatiens* (Geuten *et al.*, 2006) have also been shown to be independent of early B gene expression.

A model for the evolution of the petal identity pathway has recently been proposed (Drea *et al.*, 2007). It states that the ancient role of B class genes might have been to specify regional identity, rather than organ identity. The model is supported by functional studies of B class genes in the grass family, in another monocot species *Asparagus*, and in Ranunculales. The B class genes determine the identity of second whorl organs (lodicules) in grasses, although the morphology of the lodicules is very distinct from the morphological characteristics of petals (Ambrose *et al.*, 2000; Nagasawa *et al.*, 2003; Whipple *et al.*, 2007). In *Asparagus*, both perianth whorls contain sepal-like organs but B class genes are still exclusively expressed in the second whorl (Park *et al.*, 2003). The same holds in the Ranunculales species *Aquilegia*, in which B class genes are expressed in the second whorl but not in the petaloid organs in whorl one (Kramer *et al.*, 2007). This highlights the regional role of B genes. Studies in another Ranunculales species, the opium poppy, provide the first functional evidence based on transgenic plants that paleo*AP3* genes also determine petal identity (Drea *et al.*, 2007). These authors further propose that the ability of B class genes to function in second-whorl regional specification might have facilitated a re-recruitment of these genes, possibly through gene duplication events, for specifying petal identity multiple times during evolution.

Within the core eudicots, the euAP3- and PI-like proteins function as obligate heterodimers, which bind to their own promoters and thereby establish a positive autoregulation control (Riechmann *et al.*, 1996a; Schwarz-Sommer *et al.*, 1992). This has been suggested to be the mechanism for restricting B gene expression to petals and stamens (Theissen and Melzer, 2007). A greater variability in expression, biochemistry and function of AP3 and PI homologs has been observed outside core eudicots (Kramer and Irish, 2000; Zahn *et al.*, 2005b). In gymnosperms, homodimerization seems to be predominant, and thus homodimerization has been postulated to be the ancestral form of class B proteins. As some monocot PI homologs have the capacity to bind to DNA as homodimers (Winter *et al.*, 2002; Kanno *et al.*, 2003; Tsai *et al.*, 2008), the facultative homodimerization in monocots may represent a transitory state between obligate homodimerization in gymnosperms and obligate heterodimerization in core eudicots (Zahn *et al.*, 2005b). In the case of *Tulipa*, *Lilium*, and an orchid species *Phalaenopsis*, at least, the ability of B class proteins to form both hetero- and homodimers correlates with the broader expression domain (Winter *et al.*, 2002; Kanno *et al.*, 2003; Tsai *et al.*, 2008). Thus, the development of obligate heterodimerization and positive autoregulation loops may be connected with the function of B class genes in sharply restricted regional domains within the core eudicot flowers.

1.2.5 TM6-lineage genes

The function of *TM6*-lineage members is still poorly understood compared to the wealth of data from studies on eu*AP3*-lineage genes, mostly because the model species *Arabidopsis* and *Antirrhinum* have lost their *TM6*-type genes. The *TM6*-lineage genes represent the ancient form of *AP3*-type genes since they have the C-terminal paleo*AP3*-

domain. The intriguing difference of the paleo*AP3*- and eu*AP3*-domains has raised interests to study the *TM6*-type of genes more closely.

The most profound studies on *TM6*-lineage genes have been conducted in species of the Solanaceae family, petunia and tomato. In petunia, the loss-of-function of the eu*AP3* gene *PhDEF* caused phenotypic effects only in the petals but not in the stamens, whereas *phdef phtm6* double mutation caused homeotic changes in both petals and stamens. These results show that *PhTM6* is able to specify stamen identity independently without *PhDEF* (Rijpkema *et al.*, 2006). In tomato, a mutation in the eu*AP3* gene *TAP3* alone caused homeotic conversion of both petals and stamens, whereas the RNAi induced loss of *TM6* function caused changes only in the stamens (de Martino *et al.*, 2006). Both studies indicate that *TM6*-lineage genes have a role in stamen development. However, the petunia *PhDEF* and *PhTM6* act more redundantly, whereas the loss-of-function of either *TAP3* or *TM6* per se caused homeotic conversions in the stamens of tomato. Intriguingly, although both studies concluded a stamen-specific function for these genes, ectopic overexpression of *PhTM6* and *TM6* in *phdef* and *tap3* mutant backgrounds, respectively, showed them to be capable of fully complementing the lack of eu*AP3* function in both petals and stamens (de Martino *et al.*, 2006; Rijpkema *et al.*, 2006). Thus, the specialization of their function is most probably due to changes in the *cis*-regulatory regions and not in the coding regions. Indeed, a comparison of the upstream regulatory sequences between the *TM6*- and eu*AP3*-lineage genes of petunia and tomato revealed indicative alterations (Rijpkema *et al.*, 2006).

Table 2 *Expression of the TM6-lineage genes studied to date. The number of pluses (+) approximates the reported expression level. For poplar, the expression in sepals and petals is marked with* [a] *to indicate that there are no true sepals or petals in poplar, but a perianth cup derived from a fusion of perianth parts. nr indicates that expression has not been reported.*

Species	Gene	Non-floral	Sepal	Petal	Stamen	Carpel	Ovule	Reference
Petunia hybrida (petunia)	*PhTM6*	-	+	+	+++	+++	++	Vandenbussche *et al.* 2004
Solanum lycopersicon (tomato)	*TM6*	+	-	++	+++	+++	++	Pnueli *et al.* 1991, and 1994
Gerbera hybrida (gerbera)	*GDEF1*	-	+	+	+++	++	+	Yu *et al.* 1999
Vitis vinifera (grapevine)	*VvTM6*	+	-	+++	++	++	+	Poupin *et al.* 2007
Rosa rugosa (rose)	*MASAKO B3*	-	-	++	++	-	nr	Kitahara *et al.* 2001
Populus tricocarpa (poplar)	*PTD*	-	+[a]	+[a]	+++	-	+	Sheppard *et al.* 2000
Carica papaya (papaya)	*CpTM6-1*	-	+	++	+++	+	nr	Ackerman *et al.* 2008
	CpTM6-2	+++	+	++	+++	-	nr	

Functional studies based on mutants or transgenic plants have not been reported for *TM6*-lineage members in any other species outside the Solanaceae family except gerbera, as reported later in this study. The expression of *TM6*-type genes has been studied in diverse species, and so far all of them have been detected in petals and stamens (Table 2). However, in most cases the petal expression is not consistent throughout petal development as is typical for the eu*AP3*-type genes. The majority of the *TM6*-lineage genes are expressed in the carpels and ovules, and three of them show expression in vegetative tissues as well. In tomato and grapevine, *TM6*-like genes showed expression during fruit growth and ripening (Busi *et al.*, 2003; Poupin *et al.*, 2007). In conclusion, the *TM6*-lineage members are expressed in a more variable set of organs than are the eu*AP3*-type genes.

1.3 TCP genes in plant development

1.3.1 Regulation of floral symmetry

Diversification of floral morphologies often emerges at the level of organ elaboration in addition to the level of organ identity alterations. The TCP transcription factors have been associated with several morphological innovations and diversifications of plant forms. TCP genes regulate diverse processes in plant development, such as the differentiation of shape and size in floral organs and leaves (reviewed by Cubas, 2004; Barkoulas *et al.*, 2007), and vegetative branching patterns (Doebley *et al.*, 1997; Takeda *et al.*, 2003; Aguilar-Martinez *et al.*, 2007). Thus, in addition to the homeotic selector genes (MADSes), TCPs provide a highly interesting group of genes for evo-devo studies.

The shape of angiosperm flowers can be classified as radially symmetrical (actinomorphic, polysymmetrical), bilaterally symmetrical (zygomorphic, monosymmetrical), or asymmetrical with no symmetry planes (Endress, 2001b). Radial symmetry is the ancient form of flowers, and bilateral symmetry has evolved several times independently in separate plant lineages (Luo *et al.*, 1996; Cubas, 2004). Some of the largest angiosperm families, such as Asteraceae, Fabaceae and Orchidaceae have predominantly zygomorphic flowers, which indicates that the bilaterally symmetrical shape of flowers has been a key innovation leading to adaptive radiation. Distinct floral shapes may enable specialized plant-pollinator-interactions, enhanced out-crossing and thereby speciation (Cubas, 2004; Endress, 2001b).

The involvement of TCP transcription factors in regulating floral symmetry was first shown in *Antirrhinum*, which belongs to the order Lamiales. Wild type *Antirrhinum* flowers are bilaterally symmetrical along the dorsoventral axis. There are five petals with distinct shapes: two large dorsal (adaxial) petals, two lateral petals and one ventral (abaxial) petal. Of the five stamens, the dorsalmost is aborted during development and called a staminode. Analysis of *Antirrhinum* mutants that have radially symmetrical (peloric) flowers led to identification of *CYCLOIDEA* (*CYC*) and *DICHOTOMA* (*DICH*) genes, which both encode TCP transcription factors (Luo *et al.*, 1996, 1999). The *CYC* and *DICH* genes are needed together for defining the shape of the dorsal and lateral petals and for attaining the bilateral symmetry of *Antirrhinum* flowers. The *cyc dich* double mutant is fully radially symmetrical with all the petals showing ventral identity. In addition, the number of organs is increased from five to six in the three outer floral whorls and all the stamens are functional.

Single mutant phenotypes reveal the partial redundancy and partial sub- or neofunctionalization of *CYC* and *DICH*. In the *dich* single mutant, the number of floral organs does not change and only the dorsal petals are affected. In contrast, *cyc* single mutants have extra organs and the shapes of all the petals are affected, though the flower is still only semipeloric with dorsal petals having some lateral characteristics. Thus, *DICH* seems to have a separate role in determining symmetry within the dorsal petals, whereas *CYC* has an independent role in restricting the number of floral organs. Both *CYC* and *DICH* are expressed early in the dorsal region of floral meristems and then later in the dorsal petals and the staminode, in a partially overlapping pattern (Luo *et al.*, 1996, 1999).

CYC and DICH have been shown to interplay with two *Antirrhinum* MYB-type transcription factors, DIVARICATA (DIV) and RADIALIS (RAD). DIV acts as a determinant of ventral petal identity, and ventralization of petals in the *cyc dich* double mutant shows that CYC and DICH are needed to restrict DIV function to the ventral domain in wild type flowers (Almeida *et al.*, 1997; Galego and Almeida, 2002). This restriction occurs at the posttranscriptional level as *DIV* transcripts are detected in all petals. Both CYC and DICH activate *RAD* expression in the dorsal parts of floral meristems and flowers (Corley *et al.*, 2005). *RAD* has been shown to be a direct target of CYC (Costa *et al.*, 2005). RAD then acts posttranscriptionally to inhibit DIV in the dorsal parts of flowers, by possibly competing for common DNA or protein targets (Corley *et al.*, 2005).

Participation of *CYC*-like genes in floral symmetry regulation has been confirmed in other Lamiales species closely related to *Antirrhinum*, such as *Linaria vulgaris* and *Mohavea confertiflora*. The naturally occurring peloric phenotype of *Linaria* was caused by a heritable methylation of the *Linaria CYC* homologue *LCYC* (Cubas *et al.*, 1999a). Morphological differences between flowers of *Mohavea* and *Antirrhinum* were shown to correlate with altered expression patterns of the *Mohavea CYC* and *DICH* orthologs *McCYC* and *McDICH* (Hileman *et al.*, 2003). *McDICH* was not expressed in the dorsal petals, correlating with a high degree of internal petal symmetry, whereas the expression of *McCYC* and *McDICH* in stamens was extended, correlating with the abortion of both dorsal and lateral stamens. Thus, changes in the pattern of *McCYC* and *McDICH* expression have probably contributed to the evolution of the derived flower morphology of *Mohavea*, most likely by altering the pollination strategy.

The involvement of *CYC*-like genes in controlling floral zygomorphy has also been shown in plant lineages distantly related to Lamiales, such as in Fabaceae, Brassicaceae, and Poaceae (Citerne *et al.*, 2006; Feng *et al.*, 2006; Busch and Zachgo, 2007; Wang *et al.*, 2008; Yuan *et al.*, 2009). This indicates that *CYC*-like genes have been repeatedly and independently recruited for the regulation of floral shape. It has been suggested that the asymmetric expression pattern of *CYC*-like genes is an ancient character that might have been used several times for shape differentiation (Cubas *et al.*, 2001). Support for this idea was found from the expression pattern of the *Arabidopsis CYC* homolog *TCP1*. Although *Arabidopsis* flowers are radially symmetrical, expression of *TCP1* is transiently asymmetrical and restricted to the dorsal domain of young floral and axillary meristems (Cubas *et al.*, 2001).

1.3.2 Phylogenetics and functional diversity of TCP genes

The abbreviation TCP comes from the three founder members of the gene family: *TEOSINTE BRANCHED1* (*TB1*) from maize, *CYC* from *Antirrhinum*, and *PROLIFERATING CELL FACTOR* (*PCF*) from rice (Cubas *et al.*, 1999b). All the gene family members share a TCP domain, which is a plant-specific 59-amino acid basic-Helix-Loop-Helix (bHLH) domain that allows DNA-binding and protein-protein interactions (Kosugi and Ohashi, 1997, 2002; Cubas *et al.*, 1999b). In addition, some of the TCP proteins contain a second conserved region called the R-domain, which is rich in polar amino acids and putatively forms an α-helical structure (Cubas *et al.*, 1999b).

Based on sequence comparisons, the TCP family can be divided into two subfamilies, class I and class II or TCP-P and TCP-C, respectively (Cubas *et al.*, 1999b; Cubas, 2002; Palatnik *et al.*, 2003; Navaud *et al.*, 2007). Class I includes *PCF*-like genes, and class II the clades of *CYC/TB1*-like and *CINCINNATA*-like (*CIN*) genes. TCP transcription factors are ancient proteins. Although the *CYC/TB1*-like genes have only been found in angiosperms, both *PCF*-like and *CIN*-like genes are also present in basalmost land plants (Floyd and Bowman, 2007; Navaud *et al.*, 2007). A unifying theme, and also the putative ancient function, seems to be the regulation of growth by controlling cell division.

Although the *PCF* subfamily has the highest gene number, their functions are still largely unknown. The rice *PCF1* and *PCF2* were identified as proteins capable of binding to the promoter of *PCNA* gene, which is involved in DNA replication and cell-cycle control (Kosugi and Ohashi, 1997). Similarly, the *Arabidopsis PCF*-type TCP protein AtTCP20 activates an *Arabidopsis PCNA* homolog by binding to specific *cis*-elements that are also found in numerous other cell cycle-related and ribosomal protein encoding genes (Tremousaygue *et al.*, 2003; Li *et al.*, 2005). *AtTCP20* function is most probably redundant for some other *PCF*-like genes (13 in *Arabidopsis*), as the *AtTCP20* knockout mutants do not have a growth phenotype. Another *PCF*-like gene *AtTCP14* has been shown to have a knockdown phenotype with delayed germination and wild type function in activating germination-associated genes, such as ribosomal protein encoding genes (Tatematsu *et al.*, 2008). The general function of *PCF*-like genes has been proposed to be the co-ordinated transcriptional activation of both cell-cycle and protein synthesis genes (Tremousaygue *et al.*, 2003; Li *et al.*, 2005; Tatematsu *et al.*, 2005). Thus, they provide a link between the control of cell-cycle and cell growth in actively dividing cells.

The class II TCP genes have been shown to have mainly negative effects on growth, in contrast to the class I TCP genes. The early function of *Antirrhinum CYC* is to retard growth and reduce the number of organ primordia in dorsal parts of the floral meristem (Luo *et al.*, 1996). Moreover, *CYC* has been shown to repress the expression of a cell-cycle gene *cyclin D3b* specifically in the abortive dorsal staminode (Gaudin *et al.*, 2000). *TB1*-like genes have been identified as reducers of axillary bud growth, as first recognized in maize through the identification of the *tb1* mutant that manifests enhanced vegetative branching. The *TB1* gene is a major contributor to the less-branched domesticated maize phenotype, in which the level of *TB1* expression is twice as strong as that of teosinte, the highly branching wild ancestor of maize (Doebley *et al.*, 1997). *Arabidopsis* has two *TB1*-like genes, *BRANCHED1* (*BRC1*) and *BRC2* (*TCP18* and *TCP12*, respectively), which both act in preventing axillary bud outgrowth (Aguilar-Martinez *et al.*, 2007).

The *CIN*-like genes are associated with negative regulation of leaf growth, particularly the cell-cycle arrest related to the transition from proliferative to expansive leaf growth (Nath *et al.*, 2003; Palatnik *et al.*, 2003). Interestingly, the *Antirrhinum CIN* exerts an antagonistic effect on petals where it promotes growth, which suggests either a different pattern of growth control or a different mode of *CIN* action in petals and leaves (Crawford *et al.*, 2004). Similarly, *CYC* has a promoting effect on later growth in dorsal petals, in contrast to the earlier function in restricting meristematic growth (Luo *et al.*, 1996). In addition to growth, *CIN* also affects the differentiation of conical cells in petals, with putative interaction partners including *CYC*, *DIV* and another MYB-type transcription factor *MIXTA* (*MIX*) (Crawford *et al.*, 2004).

In *Arabidopsis*, the expression of *CIN*-like genes is regulated by a microRNA (miRNA), named *miR319/JAW*. An overexpression of these (5 out of the total of 8) *Arabidopsis CIN*-like genes occurred only by mutating the miRNA target site (Palatnik *et al.*, 2003). The *Arabidopsis CIN*-like genes have been shown to control the formation of shoot meristem and morphogenesis of shoot lateral organs. They do this by acting redundantly as negative regulators of boundary-specific *CUP-SHAPED COTYLEDON* (*CUC*) genes that belong to the family of NAC domain transcription factors (Koyama *et al.*, 2007). Ectopic expression of the *CIN*-like genes causes suppression of *CUC* expression and leads to fused cotyledons (Palatnik *et al.*, 2003; Koyama *et al.*, 2007). The *CIN*-like genes exert their effect on *CUC* genes at least partially through positively regulating a miRNA (*miR164*) that in turn negatively regulates the *CUC* genes (Koyama *et al.* 2007). Thus, the *Arabidopsis CIN*-like genes are both upstream and downstream of miRNA regulation pathways. Regulation of a *CIN*-like TCP gene *LANCEOLATE* (*LA*) by *miR319* has been shown to be important for the development of compound leaves in tomato. The overexpression of *LA* due to a mutated *miR319*-binding site results in small simple leaves with entire leaf margins (Ori *et al.*, 2007). The *miR319* target sites are also present in the rice *CIN*-like genes, which suggests that this regulation mechanism had already evolved prior to the divergence of monocots and dicots (Koyama *et al.* 2007).

To conclude, both class I and class II TCP genes are involved in growth regulation in various processes during plant development. Concordant with this, the TCP transcription factors are associated with several growth hormones. The *Arabidopsis* branching gene *BRC1* acts as an integrator of signalling pathways pathways that control bud dormancy, such as auxin and *MORE AXILLARY GROWTH* (*MAX*) related pathways (Aguilar-Martinez *et al.*, 2007). The *Arabidopsis* CIN-like protein AtTCP10 interacts in yeast with AHP proteins, which are mediators of the phosphorelay system acting in several signalling pathways, such as cytokinin and ethylene signalling (Suzuki *et al.*, 2001). Moreover, the *miR319*-regulated *Arabidopsis CIN*-like genes activate jasmonic acid biosynthesis genes, which leads to the promotion of leaf senescence (Schommer *et al.*, 2008). These findings highlight the importance of this gene family and also the demand for further research to reveal their full potential in the elaboration of plant forms.

1.4 Connections between MADS and TCP transcription factors

Both the MADS and TCP transcription factor families are connected with morphological innovations and diversification of plant forms. The genetic relationship of MADS and TCP genes is not yet clear. However, the B class MADS-box genes and TCP genes that regulate petal and stamen development seem to have several links. It has been suggested that the genetic pathways behind organ identity (MADSes) and floral meristem symmetry (TCPs) determination are parallel to each other (Kramer and Hall, 2005). It has also been proposed that TCPs are either upstream or downstream of MADS-box genes. The upstream position of TCPs was suggested because the differential expression of orchid B class genes in distinct petal types reminisces the asymmetric expression pattern of TCPs (Mondragon-Palomino and Theissen, 2008). Altered B gene expression is also linked to the peloric mutant of the orchid *Phalaenopsis equestris* (Tsai *et al.*, 2004). To date, TCP genes have not been reported in orchids.

In *Antirrhinum*, *CYC* and *DICH* are activated earlier than organ identity genes (Luo *et al.*, 1996), but later in development, the maintenance of *CYC* expression in petals was shown to be dependent on the B class MADS-box gene *DEF* (Clark and Coen, 2002). This can also be seen at the phenotypic level in a temperature-sensitive *def*-101 mutant. By altering temperature, *DEF* could be activated specifically at late developmental stages. This revealed that the restoration of dorsal petals was more pronounced than the restoration of the three lower petals of the mutant (Zachgo *et al.*, 1995). This finding reflects the connection between *DEF* and the dorsal-specific *CYC* and *DICH*. Further evidence for the upstream regulatory role of B class genes in relation to TCP genes comes from studies in rice, in which the B class gene *OsMADS2* has a *PCF*-type gene as a downstream target (Yadav *et al.*, 2007).

The functions of B genes vary during development, from the early role in organ identity to later functions in differentiation (Zachgo *et al.*, 1995; Kramer and Jaramillo, 2005). Related to the later role in organ differentiation, the B genes have been shown to activate target genes from transcription factor families that are involved in petal organogenesis, such as NAC and MYB domain genes, which have been shown to be targets for TCP genes as well. The *Arabidopsis NAP* (*NAC-LIKE ACTIVATED BY AP3/PI*) gene is upregulated directly by the B class genes *AP3* and *PI* (Sablowski and Meyerowitz, 1998). The TCP genes restrict the expression of *CUC*-type NAC genes indirectly through *miR164* (Koyama *et al.*, 2007). Moreover, the B genes directly activate an MYB gene *MIXTA*, which defines epidermal cell types in *Antirrhinum* petals (Martin *et al.*, 2002). Similarly, a *MIXTA*-like gene *AmMYBML1* that functions in specification of petal cell types has been shown to be positively regulated by B genes *DEF* and *GLO* (Perez-Rodriguez *et al.*, 2005). The *AmMYBML1* gene is positively regulated by another

MYB gene *DIVARICATA* (*DIV*), which in turn is negatively regulated by the TCP transcription factors CYC and DICH at the postranscriptional level (Corley *et al.*, 2005; Galego and Almeida, 2002; Perez-Rodriguez *et al.*, 2005). Thus, the MYB genes and the specification of petal cell types seem to connect the B genes with TCP transcription factors. Hence they also link regulation of petal identity with petal growth and shape regulation.

1.5 Regulators of inflorescence architecture

In addition to the importance of diversification of floral morphologies, varying inflorescence structures also have an essential role in the reproductive success and large radiation of angiosperms. Transition from vegetative into reproductive growth is a stepwise process in which the indeterminately growing vegetative shoot apical meristem (SAM) is first converted into the inflorescence meristem (IM) and then into the flower meristem (FM) which is determinate. Inflorescence meristems can be determinate or indeterminate, and this characteristic has been used for classifying inflorescence types. Inflorescences can be grouped into three main types (Prusinkiewicz *et al.*, 2007): racemes (indeterminate with a continuous main axis, e.g. in *Antirrhinum* and *Arabidopsis*); cymes (determinate without a main axis, e.g. in Solanaceae species such as tobacco and petunia); and panicles (determinate with a main axis, e.g. in *Sorbus aucuparia*). Additional variations on these three architectural themes are caused by altering the branching patterns (e.g. simple and compound inflorescences), internode lengths and the position of flowers.

In the simple racemose inflorescences of *Arabidopsis* and *Antirrhinum*, the genetic interplay between *LEAFY* (*LFY*) and *TERMINAL FLOWER1* (*TFL1*) in *Arabidopsis*, and the homologous genes *FLORICAULA* (*FLO*) and *CENTRORADIALIS* (*CEN*) in *Antirrhinum*, is central for patterning the indeterminate inflorescence structure. *LFY* and *FLO* are needed to promote FM identity, as shown by their characteristic mutant phenotypes in which IM produces shootlike structures instead of flowers (Coen *et al.*, 1990; Weigel *et al.*, 1992). The role of *TFL1* and *CEN* is opposite to that of the FM identity genes, as they are required to maintain the IM in an indeterminate state and their mutation causes IMs to terminate as flowers (Shannon and Meeks-Wagner, 1991; Bradley *et al.*, 1996). The *TFL1* and *CEN* genes are expressed in the centre of the IMs, where they inhibit the expression of the FM identity genes *LFY/FLO*, whereas *LFY/FLO* prevent *TFL1/CEN* expression in FMs (Shannon and Meeks-Wagner, 1993; Bradley *et al.*, 1996). The *LFY/FLO* genes encode plant-specific transcription factors with homologues found in all land plants (Maizel *et al.*, 2005). The *TFL1/CEN* genes encode phosphatidylethanolamine-binding proteins, which are putatively involved in cell-cell signalling (Bradley *et al.*, 1996). In *Antirrhinum*, the *cen* mutant shows another characteristic of interest; the terminal flower is radially symmetrical whereas the axillary flowers are bilaterally symmetrical. This feature suggests that the inflorescence architecture and production of a floral shape are connected.

Comparative studies on the *FLO/LFY* and *CEN/TFL1* homologues have revealed that changes in the function and/or expression of these genes may be associated with the generation of different inflorescence architectures (Benlloch *et al.*, 2007). For example, studies on the determinate inflorescences of tobacco suggest that the antagonism between *LFY*- and *CEN*-like genes is conserved, but the balance of their expression has altered (Amaya *et al.*, 1999). The *LFY* homologues are expressed strongly in the SAM from very early in the development instead of being confined mostly to the FM as in *Arabidopsis* and in *Antirrhinum* (Kelly *et al.*, 1995). The expression of *CEN*-like genes (those most closely related to the *Antirrhinum CEN*) is restricted from the SAM and focused on axillary meristems (Amaya *et al.*, 1999). This broader expression of the FM identity genes and more restricted expression of the *CEN*-like genes very probably relates to the formation of the terminal flowers in these inflorescences (Amaya *et al.*, 1999; Benlloch *et al.*, 2007).

1.6 *Gerbera hybrida* as a model for reproductive development

The use of *Gerbera hybrida* (gerbera) as a model organism provides the potential to address several intriguing questions for evo-devo research. The gerbera inflorescence (capitulum) is comprised of hundreds of tightly clustered flowers that have morphologically differentiated from each other so that the capitulum resembles a single large flower (pseudanthium) (Figure 2A). The presence of a condensed capitulum-type inflorescence surrounded by involucral bracts is common to the whole sunflower family (Asteraceae) and has probably been among the key innovations for the evolutionary success of this large family of over 20 000 species (Bremer, 1994). Steps in the evolutionary history of pseudanthia include: the aggregation of individual flowers, floral differentiation, and the integration of extrafloral bracts (Classen-Bockhoff, 1990). These transformation steps towards pseudanthia are most likely driven by plant-pollinator interactions that lead to a repetition of the well-established pollination pattern of single flowers.

Asteraceae capitula have been classified into several types (Gillies *et al.*, 2002) with the radiate and discoid types being the most relevant for this study. The gerbera capitulum represents a radiate type with marginal female ray florets and central bisexual disc florets, whereas the discoid capitula are comprised of disc flowers only. There are three different flower types in gerbera (ray, trans and disc) (Figure 2B), which differ in size, shape and function. The outermost ray and trans flowers are bilaterally symmetrical as the ventral and lateral petals fuse to form a large ligule while the dorsal petals are small and separate from one another. The size and shape of the flowers changes gradually along the capitulum radius so that the centrally located disc flowers are the smallest and most radially symmetrical. Ray and trans flower stamens are rudimentary and non-functional, whereas disc flowers are hermaphroditic with pollen-producing functional stamens. This variation of floral morphologies found within a single species has been among the main interests and reasons for choosing gerbera as a target for comparative developmental genetic studies.

The Asteraceae capitulum provides a beautiful example of spiral phyllotaxis in which the number of spirals follows the Fibonacci series (1, 1, 2, 3, 5, 8, 13, each number being the sum of the preceding two), which is ubiquitous in nature. The floral primordia develop in the capitulum so that the newest primordium is always approximately 137.5 ° (the golden angle) from the preceding primordium (Harris, 1991; Mitchison, 1977). This leads to two distinct left and right turning spiral rows of floral primordia (Figure 2C).

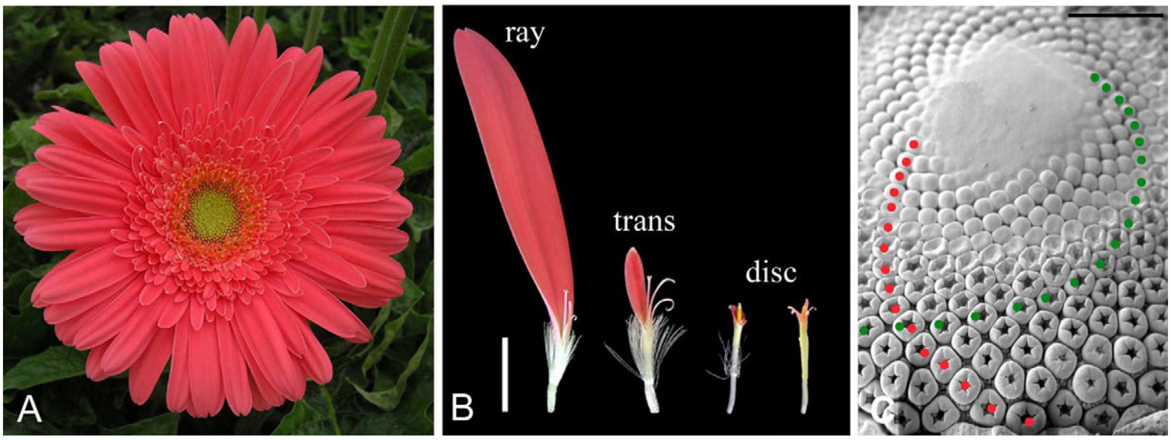

Figure 2 *The gerbera inflorescence (A) resembles a solitary flower but consists of hundreds of flowers that can be classified into three distinct types: ray, trans and disc flowers (B). The marginal ray and trans flowers are bilaterally symmetrical and female. Disc flowers are hermaphrodite and the more central their position is the closer their shape is to radial symmetry, as seen by the two disc flowers in (B), of which the rightmost is from the centre of the inflorescence. The capitulum (C) has a spiral phyllotaxis with two left and right turning spiral rows of floral primordia (examples marked with red and green spots). Scale bars 1 cm (B) and 500 µm (C).*

MADS-box transcription factors were isolated as candidate genes for gerbera flower development (collected in Table 1). The first MADSes identified from gerbera were B class genes *GDEF1*, *GDEF2*, and *GGLO1*, and C class genes *GAGA1* and *GAGA2*. By analysing expression and transgenic lines, Yu *et al.* (1999) showed that *GGLO1* functions as a classical B gene in defining petal and stamen identities, whereas both *GAGA1* and *GAGA2* define stamen and carpel identities typical of C class genes. The isolation, phylogenetic and expression analyses of *GDEF1* and *GDEF2* were also presented, and it was suggested that *GDEF2* works together with *GGLO1* in determining the B function whereas *GDEF1* was different. As no transgenic plants were reported, the comprehensive analysis of *GDEF1* and *GDEF2* functions was left for future studies. Despite the overall conservation of B and C functions, Yu *et al.* (1999) also described some characteristics specific for gerbera flower development, such as asymmetrical initiation of the B class gene expression in the outer (ventral) sides of the floral primordia before the inner (dorsal) sides. The pappus bristles, the highly modified whorl one organs of gerbera, were demonstrated to have a sepaloid origin, based on homeotic conversions detected when either *GGLO1* or *GAGA2* expression was altered.

Two of the five gerbera E class genes, *GRCD1* (*GERBERA REGULATOR OF CAPITULUM DEVELOPMENT1*) and *GRCD2*, have been shown to have whorl-specific functions. Downregulation of *GRCD1* and *GRCD2* separately caused homeotic alterations in stamens and carpels, respectively (Kotilainen *et al.*, 2000; Uimari *et al.*, 2004). In contrast, the E class genes in *Arabidopsis* (*SEP1-4*) show only vague single mutant phenotypes and have mostly redundant functions in all four floral whorls (Ditta *et al.*, 2004; Pelaz *et al.*, 2000). In addition to the loss of carpel identity, downregulation of *GRCD2* caused distinct alterations in the inferior ovaries (positioned below the whorls of floral organs) and inflorescence meristem. Inside the ovaries there were secondary inflorescences, indicating that the floral meristem had converted back to an inflorescence meristem (floral reversion). The inflorescence meristem of the capitulum was converted from the determinate into the indeterminate state, as it produced new flower meristems continuously. This shows that *GRCD2* is needed for meristem determinacy. On the whole, *GRCD2* is an important factor for terminal-meristematic functions, interconnecting floral organ identity regulation with meristem patterning and inflorescence architecture in gerbera (Uimari *et al.*, 2004).

All the gerbera studies described above are based on reverse genetics approaches, which include functional analysis of candidate genes by studying phylogenetics, expression and transgenic plants. A tool for large-scale gene expression analysis has also been developed for gerbera. The gerbera 9K cDNA microarray has been used for identifying new genes and formulating hypotheses for their functional relevance in flower development (Laitinen *et al.*, 2005, 2007, and study I). In future, the ongoing 454-cDNA-sequencing project will provide a more comprehensive set of gerbera ESTs for seeking new genes of interest. Another to date largely unexplored means for improving the power of gerbera as a model organism could be to explore the huge phenotypic diversity of gerbera cultivars developed by breeders.

2 AIMS OF THE STUDY

The general aim of this study was to gain understanding on the regulatory mechanisms behind the complex floral structures within the gerbera inflorescence. Development of distinct flower types in a compressed inflorescence is a characteristic of the Asteraceae species and cannot be studied in the main model plants, which possess only single types of flowers in their inflorescences. Thus, flower type differentiation was addressed in gerbera both in morphological and molecular terms.

To characterize the process of flower type differentiation in detail, we conducted a morphological analysis during early primordia development (I). A microarray approach was used to identify genes that are expressed differentially between ray and disc flowers and are thereby putatively involved in the specification of the flower types (I). In addition, a candidate gene approach was set up to study the role of TCP transcription factors in gerbera flower type differentiation (II). In the last part of this study, we investigated B class MADS-box genes to uncover their specific roles in defining floral organ identities in gerbera (III).

3 MATERIALS AND METHODS

The materials and methods used in this study are described in detail in the publications I, II and III, as indicated in Table 3.

Table 3 *Methods used in publications I, II and III. The publications in brackets denote that the methods were conducted only by the co-authors in the respective publications.*

Method	Publication
Histological staining and sample analysis	I
Scanning electron microscopy (SEM)	I, III
Total RNA isolation	(I), II, III
Poly(A) RNA isolation and amplification	(I)
Microarray analysis	(I)
quantitative real time PCR (qPCR)	(I), II
PCR primer design	(I), II, III
cDNA cloning	(II), III
Agrobacterium-mediated transformation of gerbera	II, III
DNA and RNA gel blot analysis	II, III
In situ hybridization	II, III
Sequence analysis and alignment	II, III
Phylogenetic analysis	(II), (III)
Yeast two-hybrid analysis	(III)

4 RESULTS AND DISCUSSION

4.1 Early stages of flower development in gerbera (I)

In order to characterize the morphological differentiation of gerbera ray and disc flowers, we performed histological and SEM analyses and divided the early phases of gerbera flower development into six stages in study I. Although the floral ontogeny in gerbera leads to clearly distinct ray and disc flowers, the formation of floral primordia and the first steps of organ differentiation are identical between the different flower types (I, Figures 1 and 2). At stage 1 the floral primordium has no visible organ differentiation. At stage 2 the petal primordia emerge by forming a ring primordium typical to Asteraceae. Then at stage 3 the pappus and stamen primordia are also visible. These first three stages are identical in ray and disc flower primordia, which can only be distinguished by their position within the inflorescence. At stage 4 the petal primordia elongate and cover the inner primordia. Elongation of pappus primordia is the general indicator of stage 5. In ray flowers, bilateral symmetry emerges through the elongation and fusion of ventrally located petals (ligule formation). In disc flowers the petals elongate evenly. Removal of petals to reveal the inner primordia showed that at this stage, the stamen primordia were already smaller in the ray flowers than in the disc flowers (I, Figure 2B, C). During stage 6, elongation of floral organs continues and differences between ray and disc flower primordia become clearer. To conclude, the critical phase for flower type differentiation is the transition from stage 3 to stage 5.

The gerbera inflorescence meristem has a low dome shape. Floral primordia initiation begins at the periphery and proceeds acropetally towards the centre of the capitulum. In general, the gerbera capitula are approximately 4 mm in diameter when the first flower primordia form, though the variation is quite extensive. Ray flowers are usually at stage 5 in capitula of approximately 12 mm in diameter, whereas the central disc flower primordia are at stage 3. However, the variation is again large and differentiation of ray flower characters may have already started before the inflorescence meristem was fully consumed by the developing disc flower primordia. Therefore, transition of ray flowers from stages 3 to 5 occurs much earlier and in smaller capitula than the transition of the central disc flowers over the same stages.

A broad morphological analysis of early capitulum development throughout the Asteraceae family by Harris (1995) revealed that the initiation of floral primordia differs between homogamous capitula (where only a single flower type is present) and heterogamous capitula (with distinct flower types). In homogamous capitula, the floral primordia initiate first in the margins and the initiation proceeds towards the apex. Thus, uniformly acropetal initiation and development correlates with homogamous heads. In contrast, in species with heterogamous capitula, the pattern of primordia initiation differs between the flower types. The disc flower primordia are the first ones to initiate and their initiation proceeds in an acropetal manner. The initiation and subsequent development of the ray flower primordia is delayed in comparison to that of the disc flower primordia and proceeds basipetally towards the margins of the inflorescence meristem.

In our study (I) we did not detect delay and basipetal pattern in the initiation of ray flowers in gerbera. In line with this finding, Harris (1995) showed that in *Mutisia coccinea*, a species closely related to gerbera (Mutisieae), the flower initiation begins and proceeds acropetally, in contrast to the other heterogamous species investigated in that study. Moreover, the organogenesis of ray flowers was only weakly suppressed compared to that of disc flowers. A careful review of the gerbera SEM data on early capitulum development revealed that a similar slight suppression of ray flower development, compared to the trans flowers next to them, also occurs in gerbera (Figure 3).

The tribe Mutisieae is considered to be basal in Asteraceae phylogeny (Kim and Jansen, 1995). The morphology and ontogeny of ray flowers described by Harris (1995) supports this interpretation. The distinction between ray and disc flowers is less clear in the Mutisieae than in the other heterogamous Asteraceae species. The ray flower shape is bilabiate as they have remnants of dorsal petals. Moreover, the pattern of primordia initiation is rather similar between the ray and disc flowers. In more derived Asteraceae species, such as *Erigeron philadelphicus*, the suppression of ray flower initiation and development is much more pronounced (Harris, 1991). To conclude, the ray flower primordia of Mutisieae are of a primitive nature, making the Mutisieae species interesting for studying the evolution of Asteraceae capitulum development.

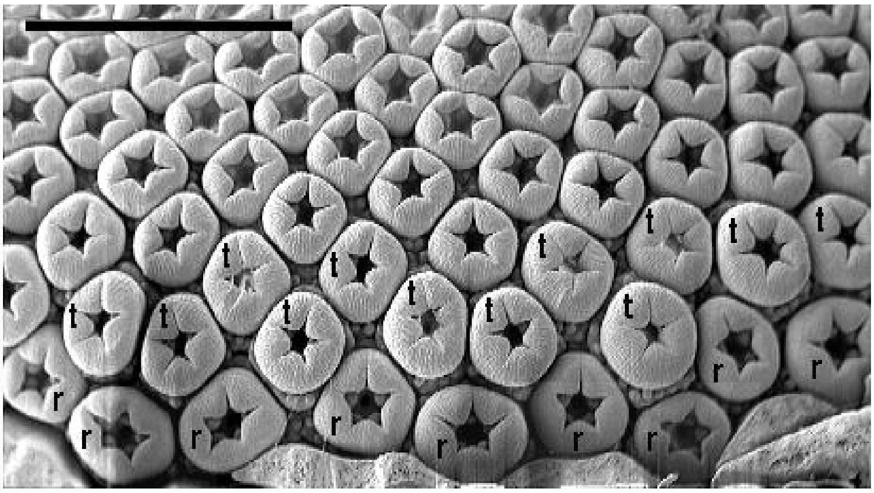

Figure 3 *Early stages of flower primordia development in the periphery of the gerbera capitulum. Organogenesis of the ray flower primordia (r) is slighty delayed compared to trans flowers (t) next to them. The petal primordia of trans flowers have elongated more than in ray flowers. Scale bar 500 µm.*

4.2 Microarray comparison of gene expression during early stages of ray and disc flower development (I)

Our first attempt to approach the mechanisms behind flower type differentiation in gerbera was through a large scale transcript profiling in study I. The morphological analyses of early stages of gerbera flower development were used to design a microarray experiment to identify genes expressed differentially in ray and disc flowers. We chose to compare the transcription between ray and disc flowers at stage 3, because at that stage the flower types are still morphologically identical. The comparison was also done at later stages 5 and 6, when differences had already occurred. The number of differentially expressed genes increased with developmental time, correlating with increasing morphological differences between the flower types. Only a very few genes showed differential expression consistently during all the stages (I, Figure 3), indicating that rapid transcriptional changes occur during early flower development.

The number of differentially expressed genes shared between stages 3 and 5 was much lower than between stages 5 and 6. This paralled the more pronounced morphological differentiation that occurred between the flower types during the transition from stage 3 to 5 over that of stages 5 to 6. Among the genes showing stronger expression in ray flowers during stages 5 and 6, there were five ribosomal protein encoding genes (I, Table 2). This suggests that the processes involving elevated ribosomal protein synthesis, such as increased protein synthesis during cell growth, are more pronounced in ray flowers at these stages. This may reflect differences seen in petal development between ray and disc flowers, such as pronounced growth of ventral petals in ray flowers.

A closer view of genes annotated as transcription factors showed that several MADS-box genes were differentially expressed in disc and ray flowers (I, Tables 3 and 4). This infers that while regulating flower organ identities and development, MADS-box genes might also take part in defining the differentiation of gerbera flower types. Most of the MADS-box genes showed differential expression at stage 6 (Table 4). Only the C-function gene *GAGA1* was also upregulated at stage 5 in disc flowers. Other MADS-box genes upregulated in disc flowers included: another C-function gene *GAGA2*, the *TM6*-like B class gene *GDEF1*, the E class gene *GRCD2* and the *AP1/FUL*-lineage genes *GSQUA1* and *GSQUA6*. Additionally, the expression of the gerbera B class genes *GDEF2* and *GGLO1* was stronger in disc flowers compared to ray flowers at stage 5 when verified by qPCR (I, Additional File 2), though the microarray data did not statistically support this.

The stronger expression detected for B and C class MADS-box genes in disc flowers compared to ray flowers is in accordance with the development of disc flowers into perfect hermaphrodite flowers with functional stamens, as B and C class genes are known to be regulators of stamen identity (Coen and Meyerowitz, 1991). However, we do not interpret the lower expression of these genes in ray flower primordia to be the cause of stamen arrest. The expression differences were detected at a rather late stage of development and thus most likely only reflect the morphological differences between the flower types. Moreover, *in situ* hybridization assays in this study (III) and by Yu *et al.* (1999) have shown that the gerbera B and C class genes are expressed in stamens of both ray and disc flower primordia. Therefore, factors acting earlier in the development are required for determining flower type identities.

In ray flowers, the *SEP*-like E class gene *GRCD1* was upregulated at stage 3 (I, Table 1), in addition to being upregulated at stage 6 (I, Table 4 and Table 4B here). Another MADS-box gene strongly upregulated in ray flowers at stage 6 was an EST *G10000100021A03* (*G1-21A3*), which is homologous to the *SOC1/TM3*-like genes. Further, two *SEP*-like genes, *GRCD3* and *GRCD5*, were upregulated in ray flowers at stage 6, in addition to *AGL12*-like EST *G0000200001C06* (*G2-1C6*). The expression of *GRCD1* and *G1-21A3* was also studied in ray and disc flowers at stage 5 by RNA blotting (data not shown). *GRCD1* showed stronger expression in ray flowers but also some detectable expression in disc flowers. In contrast, the expression of *G1-21A3* seemed to be more strictly confined to ray flowers at this stage as no signal was detected in the disc flower sample. The hypotheses for differential functions of MADS-box genes during gerbera flower type development are discussed in chapter 4.4.

Table 4 *MADS-box genes upregulated in disc flowers (A) and ray flowers (B) at stage 6 when compared to ray and disc flowers, respectively. Criterion for differential expression was an adjusted p-value with FDR < 0.05, and fold change > 1.2.*

A. Upregulated in disc flowers at stage 6			
MADS-box gene	**Description**	**p-value**	**fold change**
GAGA1	C-function	0.0173	1.9
GAGA2	C-function	0.0139	1.7
GDEF1	*TM6*-like B-gene	0.0409	1.8
GRCD2	*SEP*-like (carpel function)	0.0214	1.7
GSQUA1	*AP1*-like	0.0334	1.3
GSQUA6 (*G8-2C9*)	*FUL*-like	0.0223	1.3
B. Upregulated in ray flowers at stage 6			
MADS-box gene	**Description**	**p-value**	**fold change**
GRCD1	*SEP*-like (stamen function)	0.0174	2.5
G1-21A3	*SOC1/TM3*-like	0.0229	2.0
GRCD3 (*G2-14A7*)	*SEP*-like (*AGL6*-like)	0.0140	1.4
GRCD5 (*G7-3A3*)	*SEP*-like	0.0089	1.4
G2-1C6	*AGL12*-like	0.0365	1.4

4.3 Characterization of B class genes in *Gerbera* (III)

The function of gerbera B class genes was studied to gain understanding on the putative functional diversification within this gene group. Phylogenetic analysis placed the four gerbera B class genes into three distinct clades: *GGLO1* in the *PI/GLO*-clade, *GDEF2* and *GDEF3* in the eu*AP3*-clade, and *GDEF1* in the *TM6*-clade (III, Supplemental Figure 2). Putative orthologs of *GDEF2* and *GDEF3* were present in other Asteraceae species, which suggests an early duplication within the Asteraceae eu*AP3*-clade. Although we did not estimate the timing of this duplication, it might be related to a whole-genome duplication that has occurred at the base of Asteraceae (Barker *et al.*, 2008). Studies on MADS-box genes in other Asteraceae species have not identified more B class gene paralogs than we presented here (Dezar *et al.*, 2003; Shchennikova *et al.*, 2004), which supports our estimation on the number of B class genes in Asteraceae species. However, the possibility of further paralogs cannot be excluded.

Our functional studies on gerbera B class genes included detailed expression analyses, characterization of transgenic plants, and protein-protein interaction assays. We found that *GGLO1* was expressed exclusively in petals and stamens (III, Figures 1 and 3), which is similar to the expression of several other *PI/GLO*-clade members in asterids (van der Krol *et al.*, 1993; Davies *et al.*, 1996b). The gerbera *AP3/DEF*-clade members showed broader expression patterns. *GDEF2* and *GDEF3* were expressed weakly in pappus bristles (whorl one), carpels, and vegetative leaves, in addition to petals and stamens (III, Figures 1 and 3). Transgenic phenotypes caused by reduced *GGLO1* and/or *GDEF2* expression demonstrated that these genes perform the conventional B-function in regulating petal and stamen identities in gerbera (III, Figure 5 and Supplemental Figure 5). Reduced B gene expression caused a unique phenotype described only in gerbera thus far. In addition to the conversion of petals towards sepal (pappus bristle) identity, the abaxial side of basal petal parts had an ovary wall-like identity (III, Figures 5, 6 and Supplemental Figure 5). This provides a connection between the inferior ovaries and perianth organs, and thus supports an appendicular explanation for the origin of the ovary wall in epigynous flowers. According to the appendicular model, the outer layer of the ovary wall is comprised of fused bases of the outer floral organs (Gustafsson and Albert, 1999).

Pairwise interaction capacities of the gerbera B class proteins were studied in yeast. These analyses demonstrated that GGLO1 could form a heterodimer with all the three *AP3/DEF*-clade members (III, Supplemental Figure 7). Intriguingly, data from yeast three-hybrid assays performed in *Chrysanthemum* (Shchennikova *et al*., 2004) suggest that the two euAP3 class proteins in Asteraceae might differ from each other by their capacity to form higher order complexes. The *Chrysanthemum* B class protein CDM19 (putative ortholog of GDEF3) differs from the CDM115 protein (putative ortholog of GDEF2) when combined with the GLO-like protein CDM86 in that it does not form a ternary complex with a C class protein or with AP1/FUL-type proteins in yeast. However, our preliminary yeast three-hybrid assay that tested the same protein combinations did not find differences in the capability of GDEF2 and GDEF3 to form ternary complexes (data not shown). Thus, although differences in protein-protein interactions could explain the retention of both paralogs after duplication in *Chrysanthemum*, this seems not to be the case in gerbera.

Suppression of *GDEF1* expression caused only mild phenotypic changes, which suggests redundant roles for this gene. Although *GDEF1* was expressed in all floral organs (III, Figures 1 and 3), it was not expressed during early primordia formation in petals (III, Figure 3). Later the petal expression was weak, transient and patchy (III, Figure 3 and Supplemental Figure 3). Thus, the *TM6*-like *GDEF1* is more likely to play a role in stamens than in petals, as suggested both by its expression pattern and transgenic phenotypes with altered stamen development. Our results on *GDEF1* provide evidence that *TM6*-clade members might have a stamen-specific function outside Solanaceae as well. The mechanisms of *GDEF1* function most likely differ from the systems described for petunia and tomato. Both these Solanaceae species have two PI/GLO-like proteins, enabling partner specificity in heterodimer formation. In petunia, PhDEF interacted with both PhGLO1 and PhGLO2 in yeast, whereas PhTM6 was capable of interacting only with PhGLO2 (Vandenbussche *et al.*, 2004). In tomato, both TAP3 and TM6 showed differential binding, as TAP3 heterodimerized only with LePI, and TM6 only with TPI (Leseberg *et al.*, 2008). A similar mechanism for functional diversification cannot be suggested to occur in gerbera, where only a single PI/GLO-like protein has been identified. Differences in *GDEF1* expression between flower types (discussed below) might provide some cue for functional diversification of the *TM6*-like gene in gerbera.

4.4 A model of MADS-box gene functions in gerbera disc and ray flowers (I, III)

MADS domain proteins are known to function as dimers and higher order complexes (Egea-Cortines *et al.*, 1999; Honma and Goto, 2001). Our microarray analysis uncovered differential expression of MADS-box genes in disc and ray flowers, and led us to speculate that differential protein interactions might occur between MADS domain proteins in different flower types (I). Overlapping expression patterns are in most cases required for protein interactions *in planta* (de Folter *et al.*, 2005). To predict putative protein complexes in gerbera, we searched for transcription factors showing co-expression (standard correlation coefficient > 0.80) with the C class gene *GAGA1* and the E class gene *GRCD1*. Across the nine different microarray experiments included in the analysis, 27 genes were co-expressed with *GAGA1* and 11 genes with *GRCD1* (I, Table 5). Approximately half of these genes encoded MADS domain proteins.

The protein interaction capacities of fourteen gerbera MADS domain proteins have recently been tested by yeast two- and three-hybrid analyses (Ruokolainen *et al.*, unpublished results). These analyses support some of the protein interactions and functions hypothesized in study I, while some are not supported. For example, we suggested *GRCD4* to be a petal-specific *SEP*-like gene but the yeast two- and three-hybrid experiments do not support this. Figure 4 shows an updated version of the flower-type-specific ABC(D)E-models we hypothesized in study I (I, Figure 5).

All the four gerbera B class genes were coexpressed with *GAGA1* (I, Table 5) and upregulated in disc flowers (I, Table 3 and Additional File 2; Laitinen, 2006). The phylogenetic position and expression of *GDEF3* (III) suggests that it may be added among the other gerbera B class genes in Figure 4. The upregulation of B class genes in disc flowers most likely reflects their function in stamen development, as functional stamens are located in disc flowers. However, in the case of *GDEF1* the upregulation in disc flowers may also be explained by its differential expression in petals (III, Figures 1, 2, and Supplemental Figure 3). *GDEF1* is not expressed in petals in either flower types during primordia initiation, which suggests that it may not be required for petal identity. The later expression in disc flower petals suggests that *GDEF1* might be involved in some disc-flower-specific characteristics of petal differentiation. However, no flower-type-specific effects were detected in petals of transgenic lines showing reduced *GDEF1* expression. Thus, the putative role of *GDEF1* in disc flower petals remains uncertain.

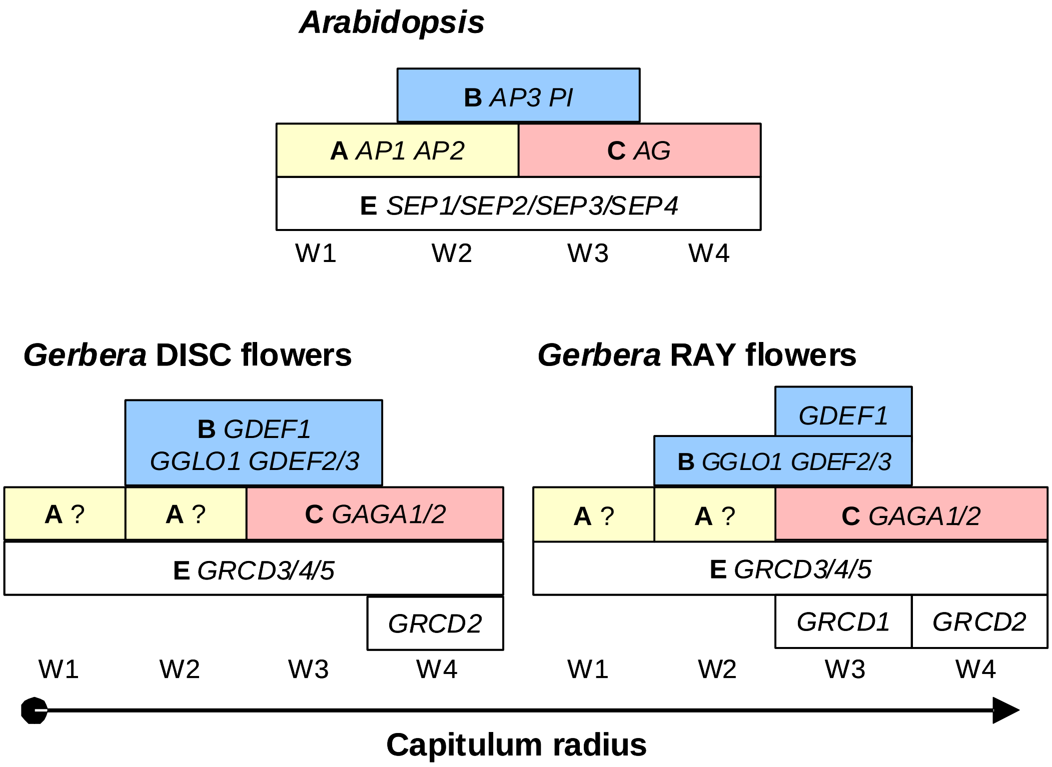

Figure 4 *MADS-box genes involved in specifying floral organ identities in four floral whorls (W1-W4) in* Arabidopsis *according to the revised ABCE-model (Theissen, 2001) are illustrated in the upper part of the figure. The lower part shows separate models for gerbera disc and ray flowers, updated from the version presented in study I.* GDEF3 *is included among the B class genes. Additionally, latest data on* GDEF1 *indicates that we have no reason to exclude it from ray flower stamens, whereas from ray flower petals its expression is mostly lacking (III). In disc flowers,* GDEF1 *may function redundantly with other B class genes.*

GRCD1 was the only gerbera MADS-box gene that was detected to be expressed differentially (stronger in ray flower primordia) as early as stage 3. This early upregulation in ray flowers implies that it may be involved in determining ray flower morphology. Transgenic phenotypes suggest that *GRCD1* regulates stamen identity specifically in ray flowers. When *GRCD1* expression is reduced, the identity of ray flower staminodia is converted to petals. In contrast, only very mild effect is detected in the functional disc flower stamens (Kotilainen *et al.*, 2000). One explanation for this phenotype is that in ray flowers, where the expression of *GRCD1* is strongest, its function is irreplaceable, whereas in disc flowers *GRCD1* might be redundant. Alternatively, the transgenic phenotype suggests that *GRCD1* determines stamen identity specifically in ray flowers, in which case it might also affect the later fate of stamens in ray flowers.

The differential expression detected for several MADS-box transcription factors led us to predict that flower-type-specific MADS protein complexes are involved in the differentiation of flower types. Further studies are needed to identify factors that act upstream of MADS-box genes and determine their differential expression along the capitulum radius. To reveal the true determinators of flower type identities, even earlier stages of gerbera capitulum development should be studied. Moreover, the cDNA probes (ESTs) of the microarray used in study I represent later stages of development. Thus, the probability that many relevant genes were lacking from the probe set is quite high. To provide a more comprehensive set of probes for future studies, we have collected samples for 454-sequencing from the earliest stages of the gerbera capitulum development. The understanding that was gained from the earlier experiments (e.g. I and II) was crucial for the sample preparation.

4.5 TCP transcription factors are involved in flower type specification (II)

We undertook a candidate gene approach to pursue regulatory mechanisms behind the specialized architecture of Asteraceae inflorescence. The CYC-like TCP transcription factors were good candidates for flower type differentiation studies, since they have been identified as regulators of floral symmetry and stamen abortion (Luo *et al.*, 1996). These are the two characteristics that differ between the gerbera flower types. Coevolution of floral symmetry and inflorescence architecture had already been suggested ten years ago (Coen *et al.*, 1995). The change of *Antirrhinum* inflorescence from an indeterminate to a determinate state in *cen* mutants also leads to altered floral symmetry. The terminal flower of the *cen* mutant is actinomorphic and resembles a peloric *cyc* mutant whereas the axillary flowers are zygomorphic as in the wild type. There is an intriguing resemblance between the *cen* mutant phenotype and the heterogamous Asteraceae inflorescences that consist of centrally located actinomorphic flowers and marginal zygomorphic flowers (Coen *et al.*, 1995).

4.5.1 The *CYC/TB1* subfamily has duplicated extensively in Asteraceae

Eight *CYC/TB1*-like genes were isolated from gerbera (II). In addition to the four full-length sequences presented in our study (*GhCYC1-4*), we obtained four distinct PCR fragments. This gives an estimate of the *CYC/TB1* subfamily size in gerbera. The size of the subfamily is parallel in sunflower (*Helianthus annuus*), in which ten *CYC/TB1*-like genes have been isolated (Chapman *et al.*, 2008). All core eudicots are thought to have three clades of *CYC/TB1*-like genes (CYC1, CYC2, and CYC3) (Howarth and Donoghue, 2006). To date no more than five copies have been identified in other species than Asteraceae. Evolution of the TCP gene family has been affected by ancient whole-genome duplications in Asteraceae. Independent paleopolyploidization events have been detected near the base of the Asteraceae and at the base of both Mutisieae and Heliantheae tribes (Barker *et al.*, 2008). Estimation of duplication times in the *H. annuus CYC/TB1* subfamily placed a duplication event approximately 40-45 MYA in all the three *CYC/TB1* clades (Chapman *et al.*, 2008). This estimation correlates with the timing of the paleopolyploidization event at the base of Asteraceae (Barker *et al.*, 2008), and hence, without any gene losses, the minimum number of *CYC/TB1*-like genes in Asteraceae is six.

Both amino acid alignment (II, Fig. S2) and phylogenetic analysis (II, Fig. S3) showed that *GhCYC1* deviates from the three other full-length sequences. We did not get statistical support for the grouping of *GhCYC1* with the CYC1 clade members *AtBRC1* (*AtTCP18*) and *LjCYC5*. However, the phylogenetic analyses conducted by Chapman *et al.* (2008) and Kim *et al.* (2008) indicate that *GhCYC1* is a member of the CYC1 clade. The gerbera *GhCYC2-4* are members of the CYC2 clade, but genes from CYC3 clade have not yet been identified in gerbera. In *H. annuus*, there are three CYC3 clade members, indicating that genes of this clade are likely to be present in gerbera as well. Moreover, as the CYC1 clade has duplicated at the base of Asteraceae, there is also most likely another *GhCYC1*-like gene in gerbera. Full-length cloning of the PCR fragments will be needed to determine their phylogenetic position in the gene family.

4.5.2 *GhCYC2* is expressed ventrally both at the inflorescence level and in single flowers

GhCYC2 was the first TCP gene recovered from gerbera. The two other CYC2 clade members were isolated only recently, and for this reason they were not included in study II. The expression of *GhCYC2* was compared between ray and disc flowers by qPCR at the same early primordia stages 3 and 5 that were used in the earlier microarray experiment (I). This revealed the upregulation of *GhCYC2* in ray flowers at both stages 3 and 5, as also confirmed by RNA blotting (II, Fig.2). None of the gerbera TCP genes were included in the microarray used in study I. Nonetheless, the genes identified by the microarray as up- or downregulated in ray flowers are potential targets for *GhCYC2*. Therefore, expression profiles of the two MADS-box genes that were upregulated in ray flowers were studied in transgenic *GhCYC2*-lines (data not shown). Neither *GRCD1* nor *G1-21A3* showed altered expression in the transgenic *GhCYC2* lines. This finding suggests that the upregulation of these MADS-box genes in ray flowers is due to some other mechanism than transcriptional activation by *GhCYC2*.

The differential expression of *GhCYC2* is specific for the early stages. At later stages *GhCYC2* was expressed similarly in petals, carpels and ovaries of both ray and disc flowers (II, Fig. 1 for ray flowers; data not shown for disc flowers). However, these RNA samples were collected from the outermost disc flowers. In the centremost disc flowers the expression of *GhCYC2* has not been studied later than at stage 7 when no expression was detected (II, Fig.2). It would be interesting to study whether *GhCYC2* expression is also excluded from the fully developed centremost disc flowers, which are closest to actinomorphy whereas most of the disc flowers are zygomorphic.

Two other recent studies on Asteraceae species have identified *CYC/TB1*-like genes to be upregulated in ray flowers. In *Senecio vulgaris*, *RAY1* and *RAY2* showed expression in young flower primordia located at the periphery of the inflorescence (Kim *et al.*, 2008). In *H. annuus*, one of the five *HaCYC2* genes (*HaCYC2c*) showed ray-flower-specific expression in RT-PCR analysis (Chapman *et al.*, 2008). Kim *et al.* (2008) devised a phylogenetic tree that combines all the *CYC/TB1*-like sequences isolated from these three Asteraceae species. It shows that *GhCYC2* is orthologous to *RAY2* and *HaCYC2e*, whereas *GhCYC3* is orthologous to *RAY1* and *HaCYC2d*. Moreover, *GhCYC4* forms a separate subclade (with low resolution) with *HaCYC2b*. The *H. annuus* ortholog of *GhCYC2* was expressed in all the studied floral parts and no difference between flower types was detected. The *GhCYC3* ortholog *HaCYC2d* showed stronger expression in ray flowers, though expression was also detected in disc flowers (Chapman *et al.*, 2008). Orthologs of *HaCYC2c*, the ray-flower-specific gene of *H. annuus*, have not been identified from gerbera or *S. vulgaris*.

In situ hybridization revealed a unique ventral pattern for *GhCYC2* expression in ray flower petals (II, Fig. 3). Outside the Asteraceae, all other CYC2 clade members characterized thus far have showed dorsal-specific expression and exerted a dorsalizing effect on flower symmetry. Studies on *H. annuus* or *S. vulgaris* did not describe whether the expression of *CYC/TB1*-like genes had specificity along the dorsal-ventral axis of single flowers (Chapman *et al.*, 2008; Kim *et al.*, 2008). Therefore, the generality of our observation remains to be confirmed in other Asteraceae species.

Support for a ventral-specific effect of *GhCYC2* comes from the analysis of transgenic plants. Transgenic gerbera lines with reduced *GhCYC2* expression had smaller ventral petals (the ligule) in trans flowers and occasionally the fused ligule was split (II, Fig. 6). In one of the lines overexpressing *GhCYC2*, disc flower petals obtained a tubular shape (II, Fig. 5), which can be seen as a ventralizing effect on petal growth. The relevance of this phenotype is emphasized by the fact that overexpression of the orthologous gene *RAY2* in *S. vulgaris* causes the formation of tubular ray flower petals (Kim *et al.*, 2008). Moreover, by overexpressing *RAY1* from *S. vulgaris* in gerbera, we obtained a line that had tubular petals in all flower types. In *S. vulgaris*, only ray flowers were affected by *RAY2* overexpression, whereas in the gerbera lines, trans and disc flower petals were also altered. This may reflect the fact that the *S. vulgaris* disc flowers are fully actinomorphic, whereas the shapes of gerbera trans and disc flower petals are more similar to that of ray flowers. Thus, they may acquire raylike characters more easily than disc flowers in *S. vulgaris*.

The transgenic phenotypes described above indicate that *GhCYC2/RAY2*-like genes may exert their ventralizing effect by promoting organ fusion. The bilaterally symmetrical shape of Asteraceae flowers is caused by the fusion and elongation of only the ventral petals, whereas in tubular flowers all the petals are fused together. Thus, the ventrally located expression we detected for *GhCYC2* correlates with the pattern of organ fusion in petals. Interestingly, the ventral-specific expression pattern is found both at the inflorescence level and at the floret level. Marginal ray flowers can be considered to be ventrally located at the inflorescence scale. Hence, the upregulation of *GhCYC2* expression in ray flowers is in line with the ventral expression of *GhCYC2* at the floret scale. The *CYC*-like genes have previously been shown to respond to a basic prepattern in a dorsal-specific manner in relation to both the vegetative and inflorescence apices (Clark and Coen, 2002; Cubas, 2004). Asteraceae species seem to have recruited *CYC*-like genes and their capacity for specific expression patterns and growth regulation in a unique way to shape their complex inflorescence structure.

4.5.3 The flower-type-specific effects of *GhCYC2*

We found that ectopic *GhCYC2* expression caused delayed growth during vegetative development (II, Fig. 4). In flowers the effect on petal growth was dependent upon flower type. Disc flower petals were longer, whereas ray flower petals were shorter than those of the wild type (II, Table S2). Other studies have also shown that TCP transcription factors have opposite effects on growth. The heterologous expression of *Antirrhinum CYC* in *Arabidopsis* causes dwarfed plants with small leaves, whereas their petals were larger due to increased cell expansion (Costa *et al.*, 2005). Mutation in *CIN*, another *Antirrhinum* class II gene, resulted in promoted growth in leaves but reduced growth in petals (Crawford *et al.*, 2004). The conflicting effects on different plant organs can be explained by organ-specific differences in growth regulatory mechanisms or in the maturation processes (Crawford *et al.*, 2004; Efroni *et al.*, 2008). Efroni *et al.* (2008) provided evidence that the primary role of the *Arabidopsis CIN* genes is to promote tissue maturation. These authors suggested that the differential effects of *CIN*-like genes in leaves and petals are due to the diverse maturation schedules in these organs. Although the ectopic *GhCYC2* expression affected growth differentially within same organs of ray and disc flowers, the development and growth of petals varies considerably between the flower types. Thus, growth control or maturation processes are also likely to vary.

GhCYC2 was identified as a factor that acts differentially between the flower types. Its expression is sequential along the capitulum radius and is activated later in disc flowers than in ray flowers. When this differential expression pattern was destroyed by ectopically induced expression, the morphological differences between the flower types became reduced. Disc flowers obtained raylike characters such as elongated ventral petals and disrupted stamen development (II, Fig. 5). We conclude that a correct pattern of *GhCYC2* expression is required for the proper differentiation of the gerbera flower types.

Two different studies have now shown that members of CYC2 clade play important roles in the regulation of flower type identities in Asteraceae. Kim *et al.* (2008) mapped *RAY1* and *RAY2* genes to the *RAY* locus, which is responsible for the natural polymorphism between radiate and discoid capitulum types in *S. vulgaris*. They also demonstrated that the *RAY* locus has been introgressed to discoid *S. vulgaris* from a closely related radiate species *S. squalidus*, and discussed the importance of introgression in providing the means to adapt to changing environmental conditions. Our study (II) provided a detailed expressional analysis for *GhCYC2* and showed transgenic phenotypes that demonstrated its role in flower type differentiation. The lack of a ray flower phenotype despite the *GhCYC2* expression being reduced was most probably due to redundancy. The two other CYC2 clade members, *GhCYC3* and *GhCYC4*, were also upregulated in ray flowers, even more specifically than *GhCYC2* (data not shown). At the floral organ level they were similarly expressed in petals, carpels and ovaries of the ray flowers (data not shown), suggesting a functional redundancy. The extensive retention of CYC2 clade members after duplications and the functions described for the members characterized so far suggest that this gene family has been important in the evolution of the complex inflorescence forms in Asteraceae.

4.6 Concluding remarks: candidate factors for the molecular basis of flower type differentiation

The pattern of primordia formation (phyllotaxis) at the shoot and inflorescence apex is known to be regulated by the growth hormone auxin. Polar auxin transport creates differential auxin concentrations in the meristems, and by this means marks the location of primordia initiation (Reinhardt *et al.*, 2003; Jönsson *et al.*, 2006; Smith *et al.*, 2006). The importance of cell-cell communications for proper phyllotaxis is highlighted by the feedback model of auxin distribution in meristems. It suggests that a newly formed primordium directs the location of the next successive primordium by affecting auxin transportation (polarity of the auxin efflux carrier PIN1) in the neighbouring cells (Jönsson *et al.*, 2006). Wounding experiments in sunflower capitula (Palmer and Marc, 1982; Hernandez and Palmer, 1988) have demonstrated the importance of cell-cell communication for proper flower type differentiation. Wounding alters positional information in the sunflower capitula and leads to the formation of extra peripheral zones. In close proximity to these wounded zones, floral primordia differentiate into involucral bracts and ray flowers.

Mechanical forces have also been suggested to be important for pattern formation (Green *et al.*, 1996; Shipman and Newell, 2005). Interestingly, several experiments have been conducted on sunflower to provide evidence for a biomechanical buckling model of primordia initiation (Hernandez and Green, 1993; Dumais and Steele, 2000). By microsurgical manipulation of sunflower capitula (initiation of cuts and fractures), Dumais and Steele (2000) were able to show that the generative region (region of primordia initiation) is specifically under compressive stress. They proposed that this compression could lead to primordium initiation in this region by means of the buckling of the tunica layer. An experiment where young sunflower capitula were physically constrained caused a formation of bracts and trumpet-shaped florets in the centre of capitula (Hernandez and Green, 1993). It was explained that the altered primordia identity was caused by increased mechanical pressure on the generative region. This suggests that flower type identity would be affected by distribution of mechanical stresses in the meristem. Another option is that compression altered the amount of space left for primordia to develop. It has been shown that the area occupied by peripheral primordia (which will differentiate into ray flowers) is about one-fifth the area occupied by primordia in the centre of the capitulum (Dosio *et al.*, 2006).

Models that combine mechanical and chemical signals have also been proposed recently (Dumais, 2007; Hamant *et al.*, 2008; Newell *et al.*, 2008). Mechanical stresses were shown to regulate orientation of microtubules in the shoot apex and thereby affect morphogenesis (Hamant *et al.*, 2008). Phyllotaxis was demonstrated to be largely independent from microtubule-reorientations and thus was suggested to be mainly an auxin-driven process (Hamant *et al.*, 2008). However, mechanical forces might be transduced at the molecular level. For example, stretch-activated ion channels or tension-induced changes in protein conformations could affect gene expression levels (Dumais, 2007). Thus, both mechanical stresses and auxin distribution may play key roles in phyllotaxis, and the relation between these two mechanisms may alter between different plant species (Newell *et al.*, 2007).

We have shown that both MADS and TCP transcription factors are expressed differentially along the Asteraceae capitulum. The gerbera *CYC*-like genes showed differential expression earlier than most of the MADS-box genes (stage 3 versus stage 6, respectively). This suggests that the *CYC*-like genes are closer to the initial specification of flower type identities, whereas the expression of MADS-box genes might only reflect the morphological differences between flower types that had been determined by earlier acting genes.

Factors that specify *CYC*-like gene expression are poorly known. Most information on the pathways upstream of *CYC/TB1*-like genes comes from studies on *BRC1* in *Arabidopsis*. The *TB1*-like *BRC1* prevents the outgrowth of the axillary buds by acting locally in the buds and integrating different regulatory pathways that control branching (Aguilar-Martinez *et al.*, 2007). The expression of *BRC1* is strongly reduced in *max* mutants, which shows that the activity of *BRC1* is controlled by the *MAX* signalling pathway. The *MAX* genes act in long-distance signalling by producing a carotenoid-derived hormone that is transported from roots to shoots (Booker *et al.*, 2004). The hormone has been recently identified to be a strigolactone (Gomez-Roldan *et al*., 2008). This signal inhibits bud growth as it reduces the capacity for auxin transport and restricts the export of auxin from the buds (Bennett *et al.*, 2006). *BRC1* is not transcriptionally regulated by auxin. However, it is required for the auxin-induced control of apical dominance (Aguilar-Martinez *et al.*, 2007). In conclusion, studies on *BRC1* show the ability of *CYC/TB1*-like genes to interact with several complex signaling pathways, as suggested by their function in diverse growth related processes during plant development.

A key to the mechanisms behind flower type differentiation in Asteraceae might come from the careful morphological studies on capitulum ontogeny conducted by Harris (1991, 1995). These studies show that formation of ray flowers correlates with the distinct phyllotaxis in the inflorescence meristem. The pattern of primordia initiation has altered both temporally and spatially in comparison to homogamous capitula in which no ray flowers form, as described in chapter 4.1. Factors that could cause a delay in primordia initiation and/or suppression of organogenesis at the meristem periphery may act as a trigger to initiate the process of ray flower differentiation and thus might be good candidates for regulators of heterogamous capitulum formation.

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