FLOWER DEVELOPMENT IN *GERBERA HYBRIDA*, ASTERACEAE

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Cover figure: Gerbera inflorescence of the anti-*GRCD1* line t3.

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, referred to in the text by their Roman numerals.

I Yu D*, Kotilainen M*, Pöllänen E, Mehto M, Elomaa P, Helariutta Y, Albert VA, Teeri TH (1999). Organ identity genes and modified patterns of flower development in *Gerbera hybrida* (Asteraceae). Plant J. 17: 51-62. *Joint first authors.

II Kotilainen M, Elomaa P, Uimari A, Albert VA,Yu D, Teeri TH (2000). *GRCD1*, a *AGL2*-like MADS box gene, participates in the C function during stamen development in *Gerbera hybrida*. Plant Cell 12 (10), 1893-1902.

III Kotilainen M, Helariutta Y, Elomaa P, Paulin L, Teeri TH (1994). A corolla- and carpel-abundant, non-specific lipid transfer protein gene is expressed in the epidermis and parenchyma of *Gerbera hybrida* var. Regina (*Compositae*). Plant Mol Biol 26: 971-978.

IV Kotilainen M, Helariutta Y, Mehto M, Pöllänen E, Albert VA, Elomaa P, Teeri TH (1999). *GEG* participates in the regulation of cell and organ shape during corolla and carpel development in *Gerbera hybrida***.** Plant Cell 11 (6), 1093-1104.

ABBREVIATIONS

bp	base pair
cDNA	complementary DNA
DNA	deoxyribonucleic acid
GA	gibberellic acid
LTP	lipid transfer protein
mRNA	messenger RNA
nsLTP	nonspecific LTP
PCR	polymerase chain reaction
RNA	ribonucleic acid

ABSTRACT

As a genetically determined structure, flower is an attractive object for developmental studies in plants. Flower development provides a good system for understanding cell differentiation and genetic mechanisms needed for organogenesis. The current molecular view on flower development has been based on studies on relatively few model species, like Arabidopsis and Antirrhinum. Research in model species, for which classical genetics is available, remains to be an important approach although it is evident that a large proportion of biological phenomena is missing from the range of variation in these species. Therefore it is important to study molecular processes behind the flower development in other species as well. Our approach for studying the central developmental phenomena is to use *Gerbera hybrida* as an experimental system. Gerbera is a member of plant family Asteraceae, which is one of the largest families in angiosperms, with over 20 000 species world wide. The Asteraceae is characterized by composite inflorescence, the capitulum, that is structurally highly adapted to insect mediated pollination. One of the major advantages of using gerbera as a model for Asteraceae is the ability to genetically transform this species.

In this study, functional analysis of the general regulation of various aspects of flower development and organ differentation has been presented. By using the regulatory genes from the MADS box family, it has been shown that the basic features of flower organ determination deduced from the model species are also present in gerbera. The characterization of genes which participate in B and C functions allowed us to use them as instruments to study the flower characteristics that are typical to Asteraceae. By using this approach it could be concluded that pappus bristles are true sepals, that B and C function genes are not participating in feminization of marginal flowers and that abortion of stamens in the marginal female flowers depends on their identity. Furthermore, genetic down regulation of expression of *GRCD1*, a member of a large *AGL2*-like MADS box gene family, revealed that it participates in determination of stamen identity during flower development in gerbera.

The development of petals, like other floral organs, is determined by genetic factors. The simple internal structure of petals and their relatively large size in gerbera ray flowers, make them a good model for studying plant organogenesis. In this study, two different strategies were chosen. First we isolated and analysed genes that were abundantly expressed in petals and secondly we attempted to to isolate genes with differential expression patterns within the petal. In addition to petals, all the clones analysed were expressed also in other floral organ. The most prominent class of genes was the one that was expressed abundantly during the development of petals and carpels. Detailed analysis of expression patterns of seven genes within the petal showed that their expression followed a basipetal pattern, thus first signal can be seen in the distal region of the petal with expression proceeding towards the proximal part of the petal. One of the most abundantly expressed genes during petal development is a gerbera lipid transfer protein (*GLTP1*). It's expression is petal and carpel specific and proceeds basipetally during petal development.

Another abundantly expressed gene during petal development is gerbera homolog of *GAST1*, *GEG*. *GEG* expression was detected in petals and carpels, with expression temporally correlating with the cessation of longitudinal cell expansion. In plants constitutively expressing *GEG*, reduced petal lengths and carpels with shortened and radially expanded stylar parts were found. Epidermal cells of both corolla and carpel are reduced in length. Radial expansion of the epidermal cells of carpel was also observed. Taken together these observations indicate that *GEG* participate in the regulation of cell and organ shape during petal and carpel development in gerbera.

abstract

Thus, asteraceous gerbera has been shown to have both concerved and derived molecular processes relative to model species in flower development. Furthermore it has proven to be a powerful system for functional analysis of general regulation of various aspects of flower development and organ differentation.

INTRODUCTION

LOGIC OF DEVELOPMENT

The divergence of plants, animals and fungi has been estimated from sequence data of several genes, have taken place around 1.6 billion years ago (Wang et al, 1999). The earliest fossil records of multicellular plants and animals known so far date from about 570-580 million years ago, sometime between 1.6 and 0.6 billion years ago parallel, independent multicellular development of two lineages, which have led to present-day plants and animals has took place (Li et al. 1999 and Xiao et al. 1999). Comparing plant development with animal development has revealed not only common developmental mechanisms, but also similarities in the logic of development. Many common principles guide development in these multicellular organisms. In their developmental programs, both plants and animals go through five similar stages, namely production of gametes, fertilization (fusion of gametes), series of cell divisions (cleavage), histodifferentation/gastrulation and organogenesis.

The major differences in their developmental programs are the two last stages, histodifferentation/gastrulation and organogenesis. As opposed to animal gastrulation which depends on cell movement, plant cells are not able to move during histodifferentation. Moreover, organogenesis continues throughout a plant's life enabling it to cope with fluctuating environmental conditions. For example immobile plants exploit existing and changing environmental conditions by introducing new organs with different functions and shapes (Walbot and Holder, 1987). Another way in which plants differ from animals is that the plant life cycle includes an independent haploid phase. The dominance of diploid and haploid phases of the life cycle varies between plant groups and generally speaking the diploid phase dominates in higher plants and the haploid phase in lower plants. At the cellular level plants have novel features compared to animals. A complex rigid cell wall consisting of many different and unique components and fibres surrounds plant cells giving them a relatively rigid shape. Also, vacuoles filled with water and water-soluble compounds can occupy 90% of the cell volume of mature cells. The major function of the vacuoles is to develop a turgor pressure and hence maintain tissue rigidity.

THE DEVELOPMENTAL PATHWAY: FROM SIGNALS TO PHENOTYPES

Developing organisms are modular in organizarion

The developmental pathway consists of individual modules which are arranged semihierarchically Raff (1996). One module can be understood as a flow of information starting with signal which is transmitted a via signal transduction pathway, which is then transmitted by gene transcriptional regulators and eventually seen as a phenotype (Figure 1). Developmental programs in metazoan development are suggested to be controlled by short tightly linked cascades of genes that force downstream genes into particular expression patterns. Many of these regulatory genes are transcription factors. For example cells which respond to external signals have receptors for them. The receptors transmit the signal via one of the many possible signalling systems. This leads to an upregulation of a chain of genes which results in the production of transcription factors which in turn determines the mode of differentiation of the receptive cell (Raff, 1996). The signals may be internal (for example a plant hormone such as gibberellic acid) or environmental (for example day length). The signal transduction pathway consists of gene products which are needed for signal production, perception, transmission and modification. Transcriptional regulators are gene products which regulate gene expression by interacting directly with the cis-regulatory elements of the target genes. Target genes are specific to the module and their

function depend on the position of the module in the developmental pathway (Doebley and Lukens, 1998; Figure 1). Thus, plant development starts with internal signals directing the modules needed to guide embryogenesis and continues with hierarchically arranged modules which are activated in the right order by the continual flow of internal and external signals. Processes, cell types, tissues or organ systems visualize the modules (Doebley and Lukens, 1998). For example, one module is needed for anthosyanin pigmentation and another regulates petal development.

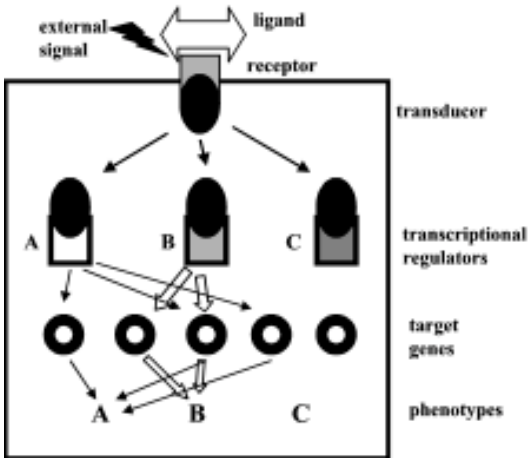

Figure 1. Simplified representation of the interactions among genes in developmental pathway. Redrawn from Doubley and Lukens (1998).

Signalling genes are pleiotrophic

Doubley and Lukens (1996) compiled a list of cloned genes corresponding to Arabidopsis mutant phenotypes with altered morphology according to their level of pleiotrophy. The mutant phenotypes were categorized from non pleiotropic (mutation afffects only a single organ or organ system) to strongly pleiotropic (mutations which strongly the disrupt overall development of the plant). In this analysis signalling genes (genes needed for signal production, perception, transmission and modification) tend to have broad pleitrophic effects, suggesting that they participate in several developmental processes and are shared between multiple developmental modules. An example of pleitrophic effects of signalling genes comes from the studies of the hormonal signalling in the maintenance of floral meristem identity. Okamuro et al. (1996) showed that *SPINDLY* (*SPY*) whose function is to repress the GA signal transduction pathway, is highly pleiotropic. Homozygous mutations in *SPY* activate a basal level of gibberellin signalling in a hormone independent manner. *SPY* is upstream of *LEAFY* (*LFY*), a transcription factor which contributes to the maintenance of floral meristem identity. *LFY* has more narrowly pleiotropic effects; homozygous *lfy* mutants are characterized by a partial and conditional block in the establishment of the floral meristem.

Transcriptional regulators are the key switches in development

The fact that transcriptional regulators are key elements in evolution has become communal wisdom among developmental biologists during recent years. The analysis of Doubley and Lukens (see above) revealed that mutations in transcriptional regulators typically have less severe phenotypic effects when compared to signalling genes. Thus, transcriptional regulators should be good targets for evolutionary modifications because alterations in their action do not have a major influence on development in general. Transcription factors are able to control very precisely coordinate the expression of their target genes. Therefore, they can act as switches which determine the phenotypic characteristics which are for example typical for each species. Transcriptional regulators could also act as evolutionary switches by providing coordinate expression of target

genes in new temporal and/or spatial contexts. This would move previously tested function into new contexts, thus giving rise for the development of novel phenotypes (Goodrich et al., 1992; Hanson et al., 1996; Doubley and Lukens, 1998).

Evolution of plant form: Cis-regulatory regions of transcriptional regulators as hotspots

Although the early pieces of evidence were suggestive, the view that changes in the temporal and/or spatial patterns of gene expression are important evolutionary mechanisms was understood already before the onset of molecular studies. Later, the importance of cis-regulatory elements in evolution has been strengthened. Cis-regulatory regions are susceptible to rearrangements implying that they could be tools for evolutionary mechanisms (Wessler et al.,1995). Moreover, modular organization of cis-regulatory regions enables a greater evolutionary flexibility (Kirchhamer et al. 1996). The expression pattern of a gene can be changed by allowing distinct elements to be added to, or removed from, its cis-regulatory regions without disrupting its other elements.

Doubley and Lukens (1998) have combined what is known of the cis-regulatory regions and transcriptional regulators to claim that they are the two key molecular mechanisms for the evolution of plant form. They have proposed that modifications in the cis-regulatory regions of transcriptional activators are the key switches in the evolution of novel forms. One of the most convincing lines of evidence for their suggestion comes from the studies of Chen et al. (1997). They showed that a spontaneous mutant of tomato, which has highly dissected leaves, is due to the fusion of a promoter of *PFP* gene in front of a *LeT6* homeobox gene. This rearrangement results in overexpression of the homeodomain protein. These results show that this kind of gene fusion can cause changes in expression patterns that lead to altered morphology. The authors suggest that such phenomena may have played a role in the evolution of the plant form. Future work on identifying and characterizing more molecular components and mechanisms needed for specific events of plant development will further test the model.

FLOWER DEVELOPMENT

Transcriptional regulators and flower development

Several universal groups of transcription regulating genes control different developmental processes during plant development. MADS box genes, *bHLH, MYB* and homeobox genes are the well-known groups which participate also in regulating flower development. The most prominent group of genes are those encoding the MADS domain containing transcriptional regulators (see below) and other major groups are *MYC (bHLH)*-, *MYB*-, and homeodomain containing proteins.

In maize, the *R*/*G* gene family of transcription are similar to the *MYC*-proto-oncogenes, now better know as the basic helix-loop-helix (bHLH) transcriptional activators, regulate the expression of biosynthetic genes needed for anthocyanin pigment formation in combination with members of another gene family (*C1*/*PI*) that are related to MYB oncogenes (Ludwig and Wessler, 1990). Also in dicot plants factors of same classes are involved in regulation of anthocyanin biosynthetic genes. In Petunia regulators of both bHLH (*JAF13*) and MYB (*AN2*) classes have been isolated and ectopic co-expression of them is suffiecient for activation of late anthocyanin gene, *DFRA*, gene and enhanged pigment accumulation (Quattrocchio et al., 1998). In gerbera, expression *DFR* is regulated according to anthocyanin pigmentation patterns in all tested varieties at several anatomical levels. Furthermore, the activity of the PGDFR2 promoter of *DFR* from the variety Regina follows the pigmentation in other

varieties which have different color patterns, thus the complex regulation of *DFR* expression occurs in trans. To identify the trans-acting regulators, a *bHLH*-type regulator, *GMYC1* was isolated and shown to have a major role in regulating DFR activity in corolla and carpel, but not in pappus and stamen. The identical patterns of *GMYC1* and *DFR* expression in corolla tissue suggest that GMYC1 also regulates *DFR* expression in a region and flower type specific manner in Gerbera (Elomaa et al., 1998). *MYC (bHLH)*-, *MYB*-, and the homeodomain containing proteins also participate in the regulation of flower development. Thus, *MYC (bHLH)*- and *MYB*-like transcription factors are key regulators of expression of flavonoid pathway genes. Flavonoids are used in multiple ways in plant development and for example in flowers they are involved in formation of pigmentation and certain flavonols are needed for pollen tube growth (reviewed by Harborne and Grayer, 1993 and Coe et al., 1981).

Homeodomain proteins, which are critical in determining posterior-anterior body axes throughout the animal kingdom, form one major universal group of transcriptional regulators in higher plants. They can be divided into five groups: HD-BELL1, HD-KNOTTED, HD-ZIP, PHD-Finger and HD-GL2 and in general, they are shown to play roles in cell specification and pattern formation (Lu et al., 1996). For example *BELL1* is considered to be involved in determining integument development and *KNOTTED* is thought to maintain the indeterminate state of apical meristems (Long et al., 1996 and Kerstetter et al., 1997). One of the best documented Arabidopsis HD-Zip gene, *ATHB-2*, is transcriptionally induced by far-red-rich light. Its gene product affects cell elongation in hypocotyls and cotyledons as well as secondary thickening in roots and hypocotyls by acting as a negative regulator of gene expression (Steindler et al., 1999). Many of Arabidopsis HD-Zip class homeodomain encoding genes are expressed during flower development. *ATHB-5, -6, -7, -8, -9, -12* and *-14* are also expressed during flower development (Söderman et al., 1994; Sessa et al., 1998; Lee and Chung, 1998). For example, besides in developing roots, leaves and cotyledons, *ATHB-6* is detected in the pistils of young flowers. The response of *ATBH6* expression to water deficiency depends on the synthesis of abscisic acid and thus it has been suggested that ATBH6 participates in the growth response of plants to drought (Söderman et al., 1999).

Lee and Schiefelbein (1999) presented beautifully how different kinds of regulatory proteins control cell patterning during specification of Arabidopsis root epidermal cell types. *WEREWOLF* (*WER*), a *MYB*-related protein has been shown to be a position dependent regulator of epidermal cell patterning during root development. Moreover *WER* has been shown to regulate the position dependent expression of a homeobox gene, to interact with a bHLH (MYC) protein and to act in opposition to a truncated MYB-like protein, CAPRICE. Thus, it is likely that different kinds of transcriptional regulators work together in controlling other developmental modules as well.

Pattern formation in flower development

Dorsoventral patterning

Dorsoventral patterning in plant development takes place when lateral organs and shoots emerge from the flanks of the shoot apical meristem (SAM). Many of these organs, like leaves and flowers may be asymmetrical in their shape. Dorsoventrality is a result of differences in the abaxial and adaxial growth of lateral SAM and its flanking regions. Dorsoventral pattern is also revealed by abaxial or adaxial specific expression of certain genes. The most interesting ones are those genes that encode putative transcription factors, thus being potentially key regulators in making the difference between the abaxial and adaxial sides; the dorsoventral pattern. The *CYCLOIDEA* (*CYC*) gene is expressed in the adaxial side of the Antirrhinum flower

primordia and in adaxially localized flower organ primordia. CYC is suggested, together with its closely related protein, DICHOTOMA (DICH), to repress the development of abaxial identity in the adaxial side of flower (Luo et al, 1996 and 1999). *CYC* and *DICH* belong to the TCP gene family, members of which encode putative transcription factors (Cubas et al., 1999). Members of Arabidopsis multigene family YABBY are suggested to promote abaxial cell fate, *FILAMENTOUS FLOWER* (*FIL*), *YABBY2* and *YABBY3* (*YAB3*) in leaf, floral meristems and all floral organs and *CRABSCLAW* in gynoecium and floral meristem, respectively (Alvarez and Smyth, 1999; Bowman and Smyth, 1999; Sawa et al., 1999; Siegfried et al., 1999). Ectopic expression of either *YAB3* or *FIL* is sufficient to induce abaxialization of lateral organs (Siegfried et al., 1999). Gene redundancy plays significant role in abaxial-adaxial patterning, such as *YABBY* gene family represents, just as the MADS box gene family does in pattern formation during flower organogenesis (see below). Abaxial-adaxial patterning in gene expression gives rise to asymmetry seen in the phenotype of many leaves and shoots. For example organ shape and cell type differences between the abaxial and adaxial sides in mature leaves ensure that the plant directs most of its light harvesting capacity towards the light (Sessions and Yanofsky, 1999).

Specification of of the fate of flower organ primordia: All you need is A, B and C functions

The wild type flowers in most of the flowering plants consist of four concentric regions called whorls. The outermost whorl, whorl one, is occupied by sepals; whorl two, petals; whorl three, stamens and the inner whorl four, carpels. The analysis of mutations affecting flower structure in Arabidopsis and Antirrhinum has led to compilation of the ABC model and identification of the genes regulating flower organ identity. The ABC model proposes that three functions, A, B and C, are expressed in adjacent overlapping domains. A function alone specifies sepal identity in wild type whorl one. The combination of A and B specifies petal identity in whorl two, the combination of B and C specifies stamen identity in whorl three. Expression of C function alone specifies carpel identity in whorl four and the determinacy of the flower meristem. Thus, the ABC model proposes that A function is present in whorls one and two, B function in whorls two and three and C function in whorls three and four (Figure 2). It also suggests that A and C functions are antagonists to each other, and that B function is restricted to whorls two and three independently of A and C functions. Analysis of mutants for B and C functions in several flowering plants are in line with the simplified, and thus excellent ABC model. This further suggests that determination of flower organ identity is conserved among flowering plants. However, the model fails to explain why loss of function mutants of B function in Antirrhinum lack the fourth whorl. This suggests that B function is, at least indirectly, needed for the formation of the fourth whorl (Tröbner et al., 1992). Problems are more severe with the A function; in fact A function mutant phenotypes are known only in Arabidopsis thus far and for example searches for A function genes by homology based searches has not been successful (Maes et al., 1998; Theißen et al., 1999). These findings suggest that A function is phylogenetically less conserved, thus problems in defining the A function reflect the quite recent and multiple origin of the flower perianth: sepals and petals (Theißen et al., 1999, see also below).

Genes providing A, B and C functions in Arabidopsis and B and C functions in Antirrhinum, respectively, have been cloned (Yanofsky et al., 1990; Jack et al., 1992; Mandel et al., 1992; Schwarz-Sommer et al., 1992; Tröbner et al., 1992; Bradley et al., 1993; Goto and Meyerowitz, 1994). In Arabidopsis, for the A function activity of both *APETALA1* (*AP1*) and *APETALA2* (*AP2*), for the B function *PISTILLATA* (*PI*) and *APETALA3*(*AP3*) and for the C function *AGAMOUS* (*AG*) is needed. In

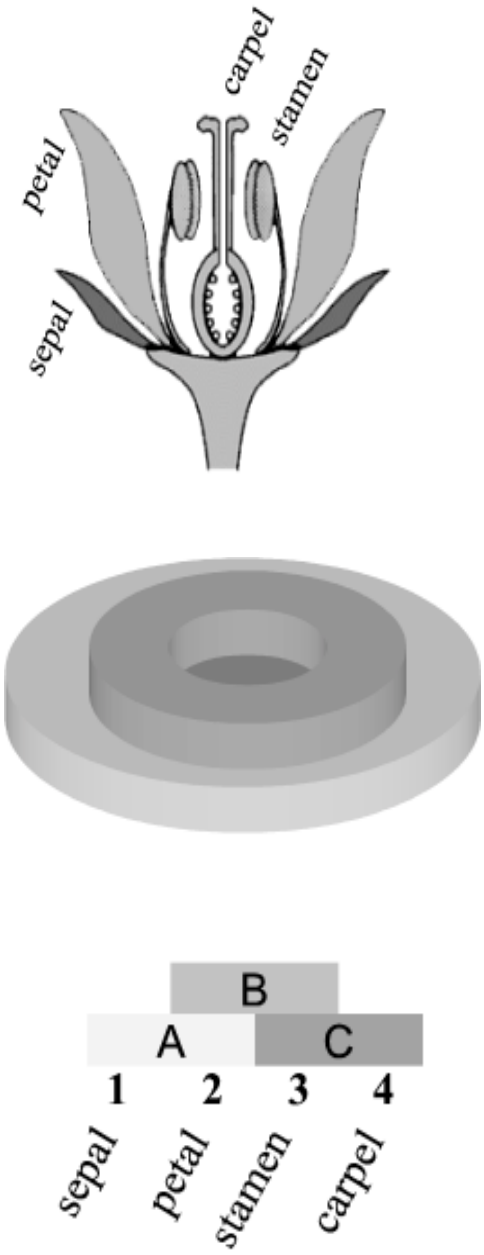

Figure 2. Schematic picture of flower structure and the ABC floral organ identity model.

Antirrhinum, the orthologous genes for the B function are *GLOBOSA* (*GLO*) and *DEFICIENS* (*DEF*) and for the C function *PLENA* (*PLE*) and *FARINELLI* (*FAR*). All of them, except *AP2* share a highly conserved, 180 bp long DNA sequence, called the MADS box. The corresponding domain in the protein is responsible for the DNA binding ability of the protein. The MADS domain proteins in seed plants are modular in their nature, and most of them consist of MADS (M), intervening (I), keratin-like (K) and C-terminal (C) domains. Thus, they are often called MIKC type MADS box genes (Münster et al., 1997). The major function of MADS domain is to perform DNA binding, but it also participates in dimerization and could be involved in binding accessory factors (Shore et al., 1997). The I region, just C-terminal of the MADS domain, largely determines the partner specificity of protein dimerization typical to MADS proteins (Riechmann et al., 1996). The K domain, which is found only in plant MADS proteins, is capable of interacting with another K domain and hence promote dimerization. The function for C domain is not known but it has been suggested to be involved in transcriptional activation or participate in formation of multimeric transcription factor complexes (Theißen et al., 1999).

Inside the (B and) C function

Phylogenetic analyses have revealed that the MADS box gene family consists of several gene groups, clades. Many clade members have highly similar sequences, share similar expression patterns and, if analysed, have highly related functions. For example, those MADS box genes that are needed for A, B and C functions fall into separate clades, namely, the SQUA group (A function), GLO and DEF groups (B functions) and AG group (C function and ovule determining genes) (see e.g. study I). Taken together, phylogenetic analyses suggest that these MADS box subfamilies have defined roles in the morphological evolution of plants (Theißen et al., 1996). But are the genes which fall into a distinct clade sufficient to perform the function the clade represents? For example, is *AGAMOUS*, the only Arabidopsis MADS box gene which fall into the AG group, sufficient for the C function in Arabidopsis? This

conclusion can be easily drawn from results which show that ectopic expression of *AG* in all whorls of Arabidopsis flower changes the identity of whorls one and two to carpels/ carpel-like organs and stamens/staminoid petals, respectively. The change in the identity of these perianth organs resemble that of *ap2*-like flowers (= loss of A function, Mizukami and Ma, 1992). The capability of AG for forming homodimers and thus performing the C function alone have been questioned by the finding that AGAMOUS can form homodimers only when the 5' ORF before the MADS box has been taken away form the construct (G. Theißen, personal communication). Furthermore a recent report on *SEPALLATA1-3* (*SEP1-3*) genes (formerly known as *AGL2*, *AGL4* and *AGL9*) shows that they are redundantly needed for B and C floral organ identity functions. *SEP1-3* genes are MADS box genes belonging to the *AGL2* clade, and genetic and protein-protein interaction data suggest that SEP proteins interact directly with the products of members of the AG, DEF and GLO groups (Pelaz et al., 2000). These results together with our findings with *GRCD1* in Gerbera presented below indicate that genes falling outside the AG, DEF, GLO and SQUA clades are needed to fulfill the A, B and C functions in different plant species.

According to the classical ABC model, the separation of identity between whorls three and four is thought to be taken care of by B class genes. However inside the C function, analyses of different *ag* mutants in Arabidopsis have revealed that different C function activities (stamen specification, carpel specification and flower meristem determinacy) can be separated. This makes it possible that the fine tuning of the C function between whorls three and four could in part be involved in the separation of the identity of whorls three and four. The molecular separation mechanism in Arabidopsis remains to be demonstrated, but two models have been proposed: The quantitative model predicts that the amount of protein needed varies for each of the functions and the qualitative model in turn proposes that AGAMOUS could make heterodimers with different partners in different functions (Sieburth et al., 1995).

Upstream of ABC: Complex regulatory interactions that establish the pattern of A, B and C gene activities

A, B and C gene activities depend on the earlier action of meristem identity genes and the patterned expression of floral homeotic genes is regulated by means of transcriptional regulation. The well studied example is the Arabidopsis meristem identity gene *LEAFY* (*LFY*), a transcription factor which is shown to be a direct upstream regulator of the floral homeotic genes *AGAMOUS*, *APETALA3* and *APETALA1* (Parcy et al., 1998; Busch et al., 1999). Parcy et al. (1998) were able to show that role of *LFY* in the meristem initiation could be separated from its role in the later activation of these floral homeotic genes. Different mechanisms are used in the activation of A, B or C function genes. The B and C function genes *AP3* and *AG* are expressed in region specific manner.

In the case of *AP3*, the combination of *LFY*, which is expressed uniformly in flower primordia, and *UFO*, which is expressed in a region specific manner in shoots and flower meristems, provide information needed. Thus, *LFY* provides flower meristem specificity and *UFO* provides the whorl two and three region specificity (Parcy et al., 1998). However, there is evidence that co-expression of *LFY* and *UFO* is not sufficient for *AP3* expression. For example in the early stages of flower primorida, in which *LFY* is expressed at high levels, constitutive expression of *UFO* fails to initiate *AP3* expression. Therefore, Lee et al. (1997) suggest that additional factors are needed for induction of *AP3* expression.

The role of *LFY* in the regulation of *AG* expression is more complex. *LFY* seems to have both a positive and a negative role in the regulation of *AG*. For example not all flowers of the strong *lfy* mutants are replaced by shoot

like organs and eventually *AG* is expressed at similar levels as in wild type flowers and in addition *AG* is expressed ectopically in the stems of *lfy* mutants. These findings indicate that *LFY* has also a negative role in the regulation of *AG* expression (Busch et al., 1999). In plants, which expressed an activated form of LFY protein, *AG* was expressed ectopically earlier and at elevated levels compared to wild type flowers. This suggests that *LFY* interacts with a region specific factor X that restricts *AG* expression to a subset of *LFY* expressing cells (whorls three and four, Parcy et al., 1998).

The expression of the C function genes, like Arabidopsis *AGAMOUS*, is restricted to the third and fourth whorls by several partially redundant factors. Mutations in genes like *AP2*, *ANT*, *CURLY LEAF*, *LEUNIG* and *STERILE APETALA* cause *AG* misexpression in various regions of the plant (Drews et al., 1991; Liu and Meyerowitz, 1995; Goodrich et al, 1997; Byzova et al., 1999 and Krizek et al., 2000). In *ap2* mutants *AG* is expressed ectopically in whorls one and two resulting in homeotic transformations of these organs into carpels and stamens, respectively (Drews et al., 1991). CURLY LEAF inhibits *AG* expression mainly in vegetative tissues and at later developmental stages also in petals (Goodrich et al., 1997).

The regulation of expression pattern of ABC genes involves the activity and cooperation of several different transcriptional regulators, and clearly illustrates how different regulators work together in controlling developmental modules. It is tempting to speculate that patterning of flower organs has co-opted the meristem patterning system (including involving *LFY* activity) when flower organs were built up to perform sexual reproduction during evolution (Parcy et al., 1998).

MADS box genes and evolution of plant development

Although the origin of MADS box genes is unclear, bacterial sequence motifs to that are homologous to the MADS domain suggest that a precursor of the MADS box like DNA binding domain was present before the separation of bacterial and eukaryotic lineages, 2-3.5 billion years ago (Martin, 1996; Mushegian and Koonin, 1996). True MADS box genes have been found in all three kingdoms; fungi, animals and plants, thus supporting the presumption that the common ancestor of these eucaryotic taxa (about one billion years ago) already had true MADS box genes (Theißen et al., 1996). A recent analysis by Alvarez-Buylla et al. (2000) suggests that duplication of an ancestral MADS box gene occurred before the divergence of plants and animals. This diversification gave rise to two main lineages of MADS box genes: types I and II. Type I lineage consist of genes encoding animal SRF like MADS domain proteins but interestingly, AGL34 like proteins, a group of plant MADS proteins without the K domain, fall into this group. Most of the plant proteins, which all have the K domain, form a group with MEF2 like sequences to form type II. Thus, this suggests that the K domain evolved after the duplication of these two lineages (Alvarez-Buylla et al., 2000). Svensson et al. (2000) isolated and analysed a MIKC type MADS box gene, *LAMB1*, from a clubmoss, *Lycopodium annotium*. Clubmosses are a sister group to other vascular plants: ferns and seed plants. Phylogenetic analyses show that *LAMB1* is situated at the base of other K box containing genes. This finding and the structural differences in sequence compared to typical K box containing MADS box genes suggests that *LAMB1* is a primitive MIKC gene. Interestingly, *LAMB1* is expressed exclusively in a reproductive structure of the moss, the strobilus, during sporogenesis. Thus it is tempting to speculate that the reproductive expression pattern of MIKC like MADS box genes, revealed by *LAMB1*, is original and ancestral (Svensson et al, 2000). Several MADS box genes have been isolated from ferns, a sister group of the seed plants. These two groups diverged about 400 million years ago. Most of the MADS box genes

isolated from the fern *Ceratopteris* have the typical structure of a seed plant MADS box gene. Their MADS domain sequence and overall domain structure are typical to MIKC type genes (reviewed in Theißen et al., 1999). Phylogenetic reconstruction analyses suggest that at least two (probably even four) different MIKC type MADS box genes already existed in the last common ancestor of ferns and seed plants (Münster et al., 1997).

Comparisons between angiosperm and gymnosperm MADS box genes have shed light on understanding the common schemes in regulation of the reproductive organs in all seed plants. As described above, the analysis of the functions of MADS box genes in Arabidopsis and Antirrhinum and establishing of the ABC model suggest that the same regulational system could be responsible for determining the flower organs in other angiosperm species. This argument is questioned also in one of the articles which make up this thesis (study I). But does the same hold true in gymnosperms? Tandre et al. (1998) have shown that *DAL*2, the ortholog of *AG* from Norway spruce, *Picea abies*, when expressed constitutively in transgenic Arabidopsis, causes developmental alterations very similar to those observed in plants ectopically expressing *AG*. Moreover, transcription of *DAL2* is restricted to the reproductive organs, the unisexual cones, especially in the developing ovule-bearing organ, the ovuliferous scale, but also in pollen cone (Tandre et al., 1998; and Sundström et al., 1999). Thus, a gymnosperm, the Norway spruce, seems to have a gene, *DAL2*, which is structurally and functionally related to angiosperm C class genes. Phylogenetic analysis and the presence of B class specific C terminal motifs in three Norway spruce genes, *DAL11*, *DAL12* and *DAL13* indicate that the genes are related to angiosperm B function genes. Specific expression of all three genes in pollen cones further support that these genes are involved in regulation of the development of the pollen bearing organs (Sundström et al., 1999). Similarly, in *Pinus radiata*, an ortholog of angiosperm B function genes, *PrDGL*, is expressed only in male cones (pollen strobili, Mouradov et al., 1999). Thus, it seems that the regulatory systems behind the development of reproductive organs of the angiosperms and the gymnosperms share common components, like B and C functions. This further suggests that B and C functions belong to ancestral functions of MIKC like MADS box genes in seed plants. More importantly, these results suggest that gene duplication gave rise to separate B and C functions in the seed plant lineage and thus was the major determinant in the transition from homospory to heterospory. The duplication and specialisation of an ancestral sporophyl identity gene into a general sporophyl gene (a C function gene) and a microsporophyll modifier gene (a B function gene) are major determinants of the development of heterospory at the evolutionary base of seed plants (Baum, 1998).

PETALS: EVOLUTION AND ORGANOGENESIS

The origin of petals

The classical view, which is also supported by the ABC model, suggests that flower organs have their closest relatives in their adjacent flower organs. In other words, the proximal whorls are each others' closest relatives (Albert et al., 1997). Based on morphological studies, petals are hypothesized to have arisen independently several times from stamens (andropetals) or from bracts (bracteopetals) in different angiosperm lineages (Takhtajan 1991). For example morphological evidence suggests that andropetaloidy has taken place several times within the lower eudicots and most basal angiosperms, such as Magnolides and Piperales, are assumed to have bracteopetals (Kramer et al., 1998; Theißen et al., 2000). However, as pointed out by Albert et al. (1997), these simple views have severe problems: for example the term "sepal" in many Magnoliales and Laurales, where floral organs

may have spiral cycles, cannot be clearly defined. Similarly, organs in the petal whorl may have structural and developmental features which are usually thought to be sepaloid. Organ terminology which is principally based on process orthology/ paralogy would always distinguish petals (AB) from sepals (A) and stamens (BC) regardless of historical or positional orthology/paralogy (Albert et al., 1997). Process orthology / paralogy here means that different structures are specified by the same type of genes (Theißen et al., 2000). Therefore study on B and C function genes which are involved in determining the organ identity in higher eudicots, should be carried in more basal groups to reveal the origin of petal. Based on the available data, two models can be drawn. The first model suggests that the ancestral condition was a single, petaloid whorl which expressed both A and B function genes and calyx whorl (sepals) was added externally (Albert et al., 1997). The second model predicts that an ancestral flower had sepaloid perianth whorl(s) and the distinction between calyx and corolla (petals) could have evolved later by the outward extension of B function into the inner whorl (whorl 2) of the perianth (Baum, 1998).

As presented above, the development of carpels and stamens was regulated by B and C function genes and most likely they contributed directly to the evolution of heterospory during seed plant development. Historically, an ancient C function gene was a general sporophyl gene and the function of an ancient B gene was to specify the microsporophyll. Quite recently, characterization of B group genes from different monocot species and basal angiosperms suggest that early expression of B group genes in petals is conserved in all angiosperms. For example if *SILKY1*, a *AP3* homolog from a monocot *Zea mays*, is mutated, lodicules (interpreted as being homologous to petals) were changed into palea like organs (one of outer sepal like organs in whorl one) and stamens into carpels (Schmidt and Ambrose, 1998). Kramer and Irish (1999) showed that in four Ranunculidae species (basal eudicots) both *AP3/DE*F and *PI/GLO* homologs were present and expressed in the youngest petal and stamen primordia. Thus, if the B genes first evolved to contribute to the evolution of heterospory, together with C genes, in the early developmental phases of the seed plants, they could be recruited to contribute to petal development, without C function, near the base of angiosperms. A key ancestral gene duplication occurred near the base the angiosperms which resulted in the distinct lineages of *AP3/DEF* and *PI/GLO* like genes (Kramer et al., 1998).

According to the ABC model petal identity is determined by the coaction of A and B functions. However, outside Arabidopsis, it seems plausible that A function may be provided by different genes or may not even exist in other species. From studies in Arabidopsis, it is also evident that the A function genes are needed for the determination of floral meristem identity. Determining flower meristem identity and thus, the separation of flower development from vegetative development is probably an evolutionarily older process than determining the identity of perianth organs in the flower. Therefore, it has been suggested that, at least in some cases, the A function is derived from the floral meristem identity function (Theißen et al., 2000). Supporting evidence comes from the finding that some orthologs of Arabidopsis *AP1* in different species are also expressed in other flower organs and in other parts of plants than just the sepals and petals (Huijser et al, 1992, work I). Thus, as a conclusion it is tempting to suggest that main part of the A function is to prevent the C function to proceed to whorl 2, and as presented above, several genes acting redundantly take care of the repression (in Arabidopsis). This in turn is needed in determining petal identity which has been derived from the stamen during evolution.

Petals, a simple model of organogenesis

In general, petals may function in three ways: attracting and assisting (i.e. providing a landing place) animal pollinators, protecting inner reproductive whorls and in some cases secreting nectars. Every different flower organ is determined by discrete molecular developmental programs, i.e. modules. As described above, these modules share common components, and thus it is useful to compare similarities and differences between petals and its neighbouring flower organs among eudicot species

The main function of sepals is to protect the inner flower parts during the early stages of flower ontogeny. In many cases the protective role is later overtaken by the larger petals but in other cases sepals may continue enlargement and later aid seed dispersal. The stamens in whorl three are the male reproductive organs of a flower and they contain the anther in which pollen is produced. Among eudicots, the initiation of all petals happens simultaneously. The same holds true with stamens but sepals have more obvious spiral phyllotaxis and are not initiated exactly simultaneously. Both petals and stamens have rapid differential growth just before anthesis (like the opening of the flower) but growth of sepals usually ceases at the early stages of their development (Albert et al., 1997; Endress, 1994). The simple anatomy of petals more resembles that of stamens than sepals. Vascular systems of both petals and stamens lack schlerencymatous cells and their vascular patterning is usually derived from the single basal trace. In contrast, sepals often have schlerencymatous cells in their vascular systems and their patterning is usually derived from three basal traces. Besides, the simple parenchyma of petals differs from that of sepals which often contains palisade parencyma layers like leaves (Albert et al., 1997). This observation is in line with the conclusion presented the previous chapter, which was based on molecular evolution studies on MADS box genes.

The simple structure of the petal and its structural and developmental similarity to the reproductive organs, make them an attractive choice for studying organogenesis. In addition, compared with vegetative organs the shapes and sizes of petals (as representative of a floral organ) are highly invariable, which is most likely due to constraints applied by plant-pollinator relationships. This regularity is advantageous for studying the basis of organogenesis in plants using genetic and molecular approaches.

FLOWER DEVELOPMENT IN ASTERACEAE

The condensed inflorescence, capitulum, is characteristic to the plant family Asteraceae (Compositae). Typically the flower organs are highly specialized and the capitulum itself is highly adapted to insect mediated pollination. The capitulum is considered to be a condensed raceme composed of typically tens or hundreds of flowers which may vary in sexuality, morphology, symmetry, anthocyanin pigmentation or organ fusion (Baaqoe, 1977; Bremer, 1994; Figure 3). So many parameters varying in a single genotype, Asteraceae is a choice beyond compare to study the molecular and genetic regulation of the developmental processes mentioned above. For example, based on analysis of certain Microseris mutants Bachmann (1991) characterized flower type specific traits and concluded that chemical gradients of morphogens lead to expression of different flower types. Another example of regulation of capitulum organization comes from studies of Palmer (1994) in which he was able to show that the phytohormone cytokinin participates in the control of flower position. Hernandez and Green (1994), in turn, have shown, by applying lateral constrains, the importance of biophysical factors in determining organization of the capitulum. All these studies show that the position of an individual flower within the capitulum is an important factor in determining its developmental fate. This indicates that signals directing the capitulum development participate in determining the fate of individual flowers.

Studies on the regulation of anthocyanin biosynthesis genes in gerbera have clarified the molecular mechanisms of gene regulation at different anatomical levels in the capitulum: between different flower types, between different flower organs and within a flower organ. Regulation of the expression of *GDFR1*, a representative of the late part of anthocyanin biosynthetic genes, senses all the different anatomical levels and the analyses of its promoter activity revealed that the spatial anthocyanin patterns are due to regulatory factors upstream of the promoter (Helariutta et al., 1995; Elomaa et al., 1998). Both anthocyanin pigmentation patterns and gene product accumulation patterns in various regions of the gerbera ray flower petal indicate region specific control of gene expression along the longitudinal axis of the petal. Studies on the anthocyanin biosynthetic genes and their regulation have shown that they form a homogenous group which is expressed in a basipetal manner during the growth of ray flower petals (Helariutta et al., 1993).

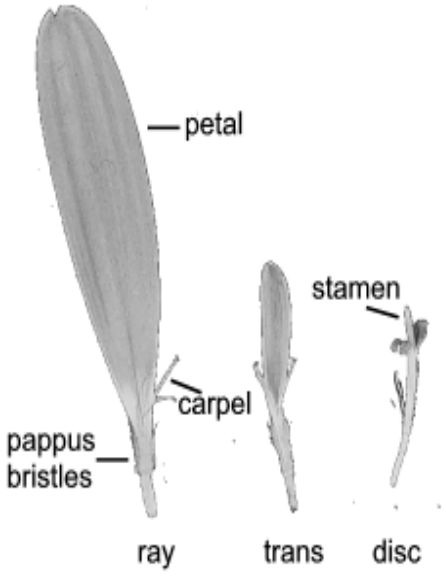

Figure 3. Different flower types of gerbera with specialized floral organs.

AIMS OF THE STUDY

Generally, the aim of the present study was to understand molecular development of gerbera flower by using two different approaches. (1) Using MADS box genes as tools to investigate the molecular determination of flower in gerbera. (2) To uncover what sorts of gene regulation strategies and gene groups are typical to late organogenesis of ray floret petal.

One approach used here is to study whether the ABC model is valid in determining the flower organ identity also in gerbera, and then taking advantage of the B and C function MADS box genes to use them as instruments to examine reproduction adaptations common to Asteraceae. The main method is to use transgenic gerbera plants either overexpressing or down regulating these genes (study I).

The same transgenic technic is used in the second approach in which is studied the role of the *GRCD1* gene, a member of the *AGL2* clade of MADS box genes, during gerbera flower development (study II). In model species, the precise functions of the members of *AGL2* clade have largely been obscure and by taking advantage the new experimental system provided by gerbera, we have been able to characterize the role of *GRCD1* in gerbera flower development, which could be applicable to other species as well.

In third approach we performed three different differential screening strategies to uncover what kind of, spatial or temporal, gene regulation strategies are typical to petal organogenesis in gerbera. In addition, we attempted to characterize what sort of gene groups are typical to late organogenesis of petals and study functions of selected genes (studies III and IV).

Specifically, in this work we have tried to answer the following questions.

(A) Is the ABC model of flower organ development ablicable to gerbera? StudyI

(B) Can we define A, B and C functions and find corresponding genes in gerbera? If yes, then can we use these genes as instruments to study flower characteristics that are typical to gerbera and the plant family Asteraceae? Study I

(C) Are the B and C function genes involved in feminisation of marginal ray flowers and does the abortion of stamens depend on organ identity or position? Study I

(D) What is the origin of pappus bristles, which are positionally orthologous to the whorl 1 organ? Study I

(E) Which developmental processes does *GRCD1* participate in? Study II

(F) Which developmental processes does *GEG* participate in? Study IV

MATERIALS AND METHODS

PLANT MATERIAL

The object in this study is *Gerbera hybrida* (2n=50-52), which is an artificial hybrid between *G. jamesonii* and *G. viridifolia*, two South African gerbera species which were crossed in the 19th century. Traditional breeding has resulted in several gerbera varieties which show differences for example in anthocyanin pigmentation patterns and in relative sizes of floral organs. The variety used in all studies is Terra regina and it was obtained from Terra Nigra BV (De Kwakel, Holland). The developmental stages of the infloresecence are described in Helariutta et al., 1993.

METHODS

Technique	used and described in
Isolation of Plant DNA and (RNA)	I, II, (III), IV
Construction of the cDNA library	I, II, (III), IV
Screening of the cDNA library	(I), II, (III), IV
(PCR cloning	I, II, IV)
(DNA sequencing	I, II, III, IV)
DNA and RNA gel blot analysis	I, II, III, IV
In situ hybridization	I, II, III, IV
(Plant transformation	I, II, IV)
Scanning electron microscopy	I, II, IV
(Phylogenetic analysis	I, II)
(Yeast two-hybrid analysis	II)
Organ and cell measurements and statistical analysis	IV

Methods which I have not used myself are braketed.

RESULTS

DEFINING THE ROLES OF B AND C FUNCTION MADS BOX GENES IN GERBERA

Six MADS box genes were isolated using several different screening strategies which were performed in order to isolate gerbera orthologs of A, B and C function MADS box genes. *GSQUA1*, *GDEF1*, *GAGA1* and *GAGA2* were isolated in low a stringency screen of a cDNA library made of young inflorescences with *DAL2*, a MADS box gene isolated from *Picea abies* (Tandre et al., 1995). *GDEF2*, and *GGLO1* were isolated from high stringency screens of petal and young inflorescence cDNA libraries, respectively, using previously amplified PCR fragments of their MADS box region (see study I). Expression (using in situ and RNA blots) and phylogenetic analysis (on combined nucleotide sequences of MADS and K boxes) were performed to determine which of these genes are potentially A, B or C function genes.

Phylogenetic analysis groups *GSQUA1* among potential A function genes (study I, Figure 2), but the expression profile of *GSQUA1* in flower differs from that of the known A function genes (whorls one and two). Its expression is detected both in the receptacle and in the developing flower bud and it resembles a continuous flow from the basis of the receptacle, through margins of the ovule, to the developing petals (work I, Figure 3 panels i and j). The pattern does not resemble that of a typical A function gene, it rather follows developing vascular system. The expression pattern of *GSQUA1* already suggests that it is not a typical A function gene. Also the expression pattern of another ABC MADS box gene differs that of the typical ABC genes. During flower development *GDEF1* is expressed at very low levels and only a faint signal is seen in the margins of corolla and stamen primordia (data not shown). In phylogenetic analysis it can be unambiguously placed in the DEF group, but inside the DEF clade its position is either unresolved or it groups with *TM6*, a *DEF* like gene from tomato with unknown function (study I, Figure 2 or data not shown). Supporting evidence for a connection between *GDEF1* and *TM6* like genes comes from the finding of the paleo*AP3*motif in the C terminus of *GDEF1* which is typical of the *TM6* lineage (Kramer et al., 1998, data not shown).

Both phylogenetic and expression analyses are in line with the conclusion that *GDEF2* and *GGLO1* are B function genes in gerbera. They are expressed in whorls two and three during flower development; in addtion, expression of *GDEF2* can be detected in developing ovary, and also in several other tissues. In a phylogenetic tree the closest orthologs of *GGLO1* and *GDEF2* are known B function genes from the *GLO* and *DEF* groups, respectively. Similarly, *GAGA1* and *GAGA2* have typical expression patterns and phylogenetic positions for C function genes. Their expression can be detected in whorls three and four and later also in the developing ovule.

From expression and phylogenetic analyses it can be predicted that *GDEF2*, *GGLO1*, *GAGA1* and *GAGA2*, besides being historical, are also functional orthologues of B and C function genes. Transgenic plants, in which the gene of interest is either overexpressed or down regulated, reveal that *GDEF2* and *GGLO1* are indeed B function genes and that *GAGA1* and *GAGA2* are C function genes. Both overexpression and down regulation phenotypes of *GGLO1* and *GAGA2* have been characterized in detail in study I. Both down regulation and overexpression of *GDEF2* give results similar to those obtained with *GGLO1* (E. Pöllänen, unpublished results). When expression of *GAGA1* is down regulated a similar, but not so prominent, identity change of whorls three and four takes place. For example, in whorl three petals develop in marginal flowers in place of staminoids, but they do not have a fused corolla ligule like in antisense *GAGA2* plants. This could be due

to some residual *GAGA2* expression left in transgenic plants where *GAGA1* is down regulated (M. Kotilainen, unpublished results, study I, Figure 6 panel k).

B AND C FUNCTION MADS BOX GENES AND MODIFIED PATTERNS OF FLOWER DEVELOPMENT IN GERBERA

After phylogenetic, expression and transgenic analyses of *GDEF2*, *GGLO1*, *GAGA1* and *GAGA2*, there is little doubt that these genes perform B and C functions during gerbera flower development. Based on these results, the first conclusion that the ABC model is also applicable to gerbera is evident. More importantly, these genes can be used as instruments to study gerbera flower characteristics which are typical Asteraceae specific adaptations of flowering.

Expression analysis of B and/or C orthologs in the dioecious plants sorrel and white campion suggests that the absence of these genes could play a role in the development of unisexual flowers. In both cases, expression of these genes came undetectable as soon as the inappropriate organs cease to develop (Hardenack et al., 1994; Ainsworth et al., 1995). In gerbera, at early stages the flower primordia development all flower types are indistinguishable and the differentiation between marginal ray/trans flowers and central disc florets takes place later. During an early organ differentiation stage of flower development anther development arrests in the marginal flowers resulting in abortion of stamens and thus feminisation of marginal flowers. Because spatial and temporal expression patterns of all B and C function MADS box genes are identical in all different flower types, it is obvious that these genes are not involved in the developmental processes that lead to anther abortion in marginal flowers of gerbera.

In whorl three of the marginal flowers stamens cease to develope, later they senescence and form staminodes. If the identity of whorl three organs was changed to petals by down regulating *GAGA2* expression, these organs do not show any signs of developmental arrest or senescence. Similarly, if *GAGA2* or *GGLO1* are overexpressed, the development of whorl two or four in marginal flowers, respectively, begin to wither resembling the development of staminodes in wild type (study I, Figures 5A and 6F). Taken together, the analysis of these transgenic lines gave molecular evidence that developmental arrest of whorl three organs in marginal flowers is dependent on the identity not the position of the floral organs.

In the plant family Asteraceae a leafy calyx is often missing and the whorl two organ is surrounded by either pappus bristles or small bract like leaves, or whorl one organs are missing completely. In gerbera pappus bristles develop in whorl one position. These simple organs consist of a single cell type, lacking e.g. vascular system and their function is to aid seed dispersal later in development. There has been a debate for some time about the origin of the pappi. Are they true sepals or do they originate from outside the flower being specialized inflorescence bracts? Again with the help of transgenic plants overexpressing or down regulating B or C function genes we can change the identity of flower organ of interest towards another. On the surface of chimeric organs in whorls two and four of the plants where *GGLO1* and *GAGA2* are down regulated, respectively, true pappus bristles emerge. According to the ABC model, the identity change should approach that of whorl one, this shows that the pappus bristles are true sepals (study I, Figure 7, panels h and j).

GRCD1 PARTICIPATES IN THE C FUNCTION

Analysis of the cDNA corresponding to *GRCD1* was performed following the same strategy as presented with the B and C function MADS box genes. Phylogenetic analysis showed that *GRCD1* belongs to the *AGL2* clade which consists of a growing number of MADS box genes isolated from

gymnosperm and angiosperm species. Interestingly, until quite recently the precise functions of these genes, which are expressed without exception during flower development, have remained obscure.

Expression of *GRCD1* in gerbera flower varies spatially over the course of development suggesting that it is regulated by several different programs and thus GRCD1 has potential to participate in several modules needed for flower development. The onset of *GRCD1* expression takes place simultaneously with the B and C function genes and, it can be detected in the ring primordia from which the perianth of flower originates. During the early stages of flower organ differentiation *GRCD1* is expressed in all four flower whorls and in the ovule. Later its expression concentrates on whorls three (stamens), outer surface of whorl four (carpels) and outer integument of ovule (study II, Figure 2). At this stage a weaker signal can be seen in developing petals which is later concentrated in the epidermal cells on the abaxial side of the petal ligule (data not shown).

All three transgenic gerbera plants, in which *GRCD1* expression was specifically down regulated, had altered whorl three identity. In marginal flowers a complete homeotic conversion had taken place; instead of staminodes developed true petals. In the central flowers the stamens remained fertile, but they had chimeric structures on their abaxial surface; stomata, which normally develop only on the abaxial surface of petals (study II, Figures 4 and 5). No other flower organs showed any differences in their development, the only exception was that in one transgenic line the shape of petals and color of abaxial side of petals was altered. This change resembled weak down regulation phenotype of B function genes (unpublished results). This phenotype spatially correlates the late expression pattern of *GRCD1* in petals; *GRCD1* expression was detected in epidermal cell layer of petal, especially on abaxial side (unpublished results, Helariutta et al., 1993) Earlier, based on expression analysis of Arabidopsis *AGL2*, *AGL4* and *AGL9* genes, it has been suggested that *AGL2* like genes could be upstream of the A, B and C function genes (Flanagan and Ma, 1994; Savidge et al., 1995 and Mandel and Yanofsky, 1998). However, in gerbera *GRCD1* expression is not affected when either *GAGA1* or *GAGA2* expression is down regulated. Similarly, down regulation of *GRCD1* expression does not affect the expression of *GAGA1* or *GAGA2* (study II, Figure 3). Because both GAGA1 and GAGA2 proteins are able to interact with a GRCD1 protein in yeast two hybrid analysis (study II, Figure 6), we propose that this pairing takes place also in planta and it is needed for C function in whorl three in gerbera.

STUDY OF ORGANOGENESIS USING PETAL ABUNDANT GENES

As stated earlier, flower organs are an excellent choice to study organogenesis because the high degree of regularity in their shape. Compared to reproductive organs, petals have a simple internal structure, though this structure resembles that of neighbouring organs. In gerbera the most prominent floral part is the ray flower bilabiate corolla. The bilabiate corolla consists of a blade-like ligule part (which in part consist of three fused petal lobes and two rudimentary ones) and a proximal tubular part, the tube (Figure 3; study IV, Figure 2).

The highly variable anthocyanin pigmentation patterns in different gerbera varieties also include differential pigmentation along the longitudinal axis of the ray flower corolla ligule. This may reflect spatially restricted gene expression patterns and gene regulation strategies in the corolla Region specific control of gene expression is further indicated by the observation of spatially restricted gene product accumulation patterns in various regions of the gerbera ray flower corolla (Y. Helariutta, personal communication). During ray flower petal development, anthocyanin biosynthetic genes form a homogenous group

which is expressed in a basipetal manner (i.e. from the distal end towards the proximal end, Helariutta et al., 1993). They are expressed mainly during the growth phase of ray flower corolla when the shape of the corolla is determined by both cell division and elongation.

To reveal what sorts of gene groups and regulation strategies are needed during late corolla organogesis we performed three distinct differential screenings. The developmental stages studied were chosen so that the time window started when the rapid growth phase was still going on, but cell division had ceased, and the window ended when the size and shape of corolla was determined. The formation of anthocyanin pigmentation is a sign of well developed vacuoles which in turn tells us that cell division is ending. Two different differential screening strategies were chosen. First, we isolated and characterized genes that are expressed abundantly in ray flower corolla. A cDNA library made from corolla mRNA was differentially screened with corolla and leaf cDNA probes made from corresponding RNA pools. In a second approach, we attempted to isolate genes of which expression is spatially restricted into a specific region of the corolla. We performed two separate experiments: cDNA libraries made from proximal and distal halves of a corolla were both screened with probes made from the first strand cDNA pools of (1) the tube region of a corolla, (2) the proximal fifth of a corolla ligule and (3) the distal fifth of a corolla ligule.

Altogether 120 000 clones were screened - 20 000 between corolla and leaf; 50 000 in each "between regions within the corolla" - experiment. Taken together none of the genes analysed were specific to corolla nor spatially restricted to a specific region of it. We analysed expression patterns of seven different genes in detail. All the genes were expressed at least in one other floral or vegetative organ. Interestingly, the most common group of genes was expressed abundantly in petals and carpels. In gerbera, carpel style is a highly elongated, nonphotosynthetic and in many varieties anthocyanin pigmented structure. *GLTP1*, *GEG* (studies III and IV), the unidentified genes *GTY37* and *GTK17*, and genes studied in another context, like *GDFR1* (Helariutta et al., 1993) and *GMYC1* (P. Elomaa, personal communication) are expressed abundantly during petal and carpel development. In both of these organs, the spatial expression pattern of these genes is restricted to the epidermal and/or parenchymatic cell types. This may indicate that in these cell types petals and carpels share similar developmental programs in gerbera. Within ray flower corolla all seven clones analysed in this study (besides anthocyanin genes *GDFR1* and *GCHS1*, Helariutta et al., 1993; Helariutta, 1995) have basipetal expression pattern, thus their expression start - and cease - from the distal end of the corolla. Maturation of corolla, visualized for example by anthocyanin pigmentation, has the same basipetal pattern further demonstrating the generality of this pattern. These studies suggest that many genetic programs could be regulated by a single basipetal signal gradient along the longitudinal axis of corolla and that different factors react to it at the signal concentration characteristic of their regulation.

Based on sequence and gene expression analyses we chose three genes from the differential screens for further studies; *GRCD1* (study II), *GLTP1* (study III) and *GEG* (study IV).

SPATIAL AND TEMPORAL EXPRESSION PATTERN OF A PETAL ABUNDANT GENE *GLTP1*

Because putative amino acid sequence of the most abundant clone isolated in differential screen between petals and leaves had many characteristics similar to putative nonspecific lipid transfer proteins, we named the gene *GLTP1* (a Gerbera nsLipid Transfer Protein 1) including an obvious signal sequence in the amino terminus and eight conserved cysteine

residues. The expression patterns vary among different *LTPs*, and they are characterized by developmental and tissue specificity with distinct expression patterns for different genes (Kader, 1997). Many *LTP* genes are expressed in the epidermis, examples of these are tobacco *LTP1* and maize *LTP*. LTP proteins, like Arabidopsis LTP and carrot EP2, have also been reported to accumulate mainly in the epidermal cell layers (Sossountzov et al., 1991; Sterk et al., 1991; Fleming et al., 1992; Thoma et al., 1993). In many cases, *LTP* genes are expressed at early stages of development of the organ in question, like tapetum specific *LTPs* in tobacco and carrot *EP2* are expressed primarily in young developing inflorescences (Koltunow et al., 1990 and Sterk et al., 1991). The expression pattern of *GLTP1* is unique among *nsLTPs*. It is specific to petals and carpels, and it can be detected both in epidermal and parenchymatic tissues of these organs. Moreover *GLPT1* expression takes place temporally in relatively late stages of petal and carpel development.

So far there is no direct evidence what the function of plant nsLTPs is. Based on localization and in vitro experimental studies several roles for nsLTPs have been proposed, including participation in cutin biosynthesis, adhesion, adaptation to various stresses, anti microbial activity etc. (Kader, 1997, Park et al., 2000). In gerbera, the petals are covered with thick cuticula, which is supposed to add surface sheen and color, in addition to aiding water economy and resistance to disease. Thus we hypothesized that GLTP1 could play a role in cuticle formation. To study the role of *GLTP1* in petal development we created transgenic plants in which *GLTP1* expression was down regulated. Two transgenic lines in which *GLTP1* expression were largely down regulated was obtained. In visual or scanning electron microscopy studies we could not detect any phenotypic change in petal structures (unpublished results).

GEG, A *GAST1* LIKE GENE, AND LATE ORGANOGENESIS OF THE GERBERA PETAL

GEG was chosen for further studies based on its expression pattern: in the first screen of the proximal ray flower corolla cDNA library, it seemed to be expressed more strongly in the proximal part of corolla than in the distal. More detailed analysis revealed an intriguing expression pattern for *GEG* during corolla maturation: just before the opening and unfolding of ray flower corolla *GEG* expression occurs almost simultaneously from both ends of the corolla. The very first signal is seen in the proximal part of corolla, in the region which joins the tube and the ligule and just after that *GEG* expression is detected in the distal end of corolla ligule. During opening of ray flower corolla, the proximal expression proceeds basipetally into the tube and acropetally into the ligule. The distal expression proceeds basipetally into middle of ligule, where both expression patterns meet just as the ray flower corolla has opened (study IV, Figure 2). *GEG* expression continues at a high level in the entire corolla until it senescences. Detailed biometric analysis of corolla growth revealed that before its opening, the corolla expands both longitudinally and laterally and soon after opening, growth ceases in both directions. Thus, *GEG* expression temporally coincides with cessation of corolla growth. At the cellular level, *GEG* expression coincides tightly with cessation of the growth of ligule epidermal cells, but only in the longitudinal direction (study IV, Figure 5). Another part of the flower in which *GEG* is expressed at high levels is the stigma+style part of the carpel. Similar to the corolla, correlation between *GEG* expression and cessation of longitudinal growth, both at organ and cellular level was detected, but in the lateral direction of epidermal cells of carpels do not expand during the elongation period or later (study IV, Figure 4). Thus, *GEG* expression tightly correlates with completion of organ and cell elongation in the longitudinal direction in both corollas and carpels.

In an attempt to go beyond correlation, towards causality in revealing the function of *GEG*, we generated transgenic gerbera plants in which *GEG* is constitutively expressed. The overexpression phenotypes of corolla and carpel, both at organ and cellular level, were in harmony with correlation data: the organs and the epidermal cells were shorter when *GEG* is constitutively expressed compared to control plants. Interestingly, in carpels, besides being shorter, the epidermal cells of the style were also wider. Because any increase in style width is not observed during endogenous *GEG* expression, the radial growth of these cells may be a secondary effect. In conclusion, the primary role of GEG is to inhibit cell expansion in the longitudinal direction and to participate in the regulation of corolla and carpel shape.

Sequence analysis revealed that *GEG* shares a high similarity to previously characterised gibberellic acid inducible genes, members of a *GAST1* gene family, the functions of which have remained obscure. Members like, *GAST1* and RSI1 of tomato, *GASA* gene family of Arabidopsis, *GIP1* of petunia, have an expression that is regulated by phytohormones like GA or auxin (Shi et al., 1992; Taylor and Scheuring, 1994; Herzog et al., 1995; Ben-Nissan and Weiss, 1996). Similarly to most of the *GAST1* gene family members, the expression of *GEG* is also stimulated by GA. Application of GA_3 upregulated *GEG* expression in detached ray flower corollas (study IV, Figure 12). The amino acid sequences encoded by all *GAST1* gene family members share similar characters. A signal peptide in the N terminus, a variable region varying in length and hydrophobicity in the middle and a highly conserved C terminal end: of these 60 amino acids, 22 are identical and 12 of these are cysteines.

DISCUSSION

All the four studies forming this thesis are discussed in the relevant scientific context in detail in the corresponding published articles. Therefore only a synopsis and selected conclusions are presented in this chapter.

POWER OF MADS GENES

In the early 90's the molecular determination mechanisms of different flower organs were characterized and the ABC model which was based on Arabidopsis and Antirrhinum mutants was created. In the beginning the communal wisdom was that the simple ABC model could be extrapolated to other angiosperms species. Therefore it became necessary for people interested in molecular development in Asteraceae, to study if the Model holds true in one of the largest and thus one of the most successful angiosperm families. As presented above, generally speaking the ABC model is applicable to gerbera and the determination of different floral organs can be explained by the identified B and C function genes. Besides an important enlargement of the ABC system, study of its components in different flowering plants enables better understanding of molecular and genetical mechanisms of plant development. An example of difficulties encountered when a simple model is taken too literally, is the early finding of an AGAMOUS homodimer and conclusion that it is sufficient for C function in Arabidopsis - and in other plant species as well. AGAMOUS homodimerization takes place only if an N terminal peptide before the MADS domain is removed, but such a proteolytic cleavage was never shown in planta. Later information from yeast two hybrid and in vitro experiments show that proteins encoded by *AGL2*-like and C class MADS genes of Antirrhinum, tomato, gerbera and Arabidopsis infact can make heterodimers. Recent functional studies of *SEP1-3* genes of Arabidopsis and *GRCD1* of gerbera complement the picture of C function showing that other (MADS box) genes are needed as well (Pelaz et al., 2000; study II). Involvement of other genes in the C function adds a new dimension to it enabling better understanding different aspects of the C function; for example in dissecting its different subfunctions.

As stated in the introduction, transcription factors are most likely key switches of development and the main driving force for evolution. What is the minimal set of flower organ determining transcription factors that guide the development of a group of competent cells to become a flower, i.e. sufficient to induce an ectopic flower? In the light of present data, it is tempting to suggest that the members of A, B, C and AGL2 clades of MADS box genes, which are common to angiosperms and gymnosperms, are enough.

POWER OF A SIMPLE MODEL

As stated above, the advantage of studying petals is clear compared to vegetative organs or to other floral organs. Organogenesis in vegetative organs, such as in leaves is more variable in the sense that environmental factors have more effect on the determination of their final shape and size. The development of reproductive flower organs is determined by genetic programs, but because of their function, they have more complex structures and developmental programs as compared to petals. From the evolution perspective, petals resemble the neighbouring floral organs and are most likely in many cases derived from stamens, thus they also provide a good model for studying floral organogenesis.

One major general result from the differential screening between different spatial regions within ray floret corollas is that all the characterized genes, representing different developmental programs, modules, could be regulated by a single basipetal gradiental signal. Factors determining different developmental modules during petal maturation, could react to different concentrations of single signalling molecule. Thus a single signal could be behind the

regulation of late morphogenesis petal in determining its shape and size.

The functions of lipid transfer proteins have remained obscure, but a quite recent interesting finding is that a LTP is involved in the lily pollen tube adhesion in in vitro bioassay (Park et al., 2000). The lily LTP is one of two stylar components that are necessary for adhesion and together they induced adhesion of pollen tubes to an artificial stylar matrix in vitro. Park et al. (2000) speculated that lily LTP could act as an adhesive agent between the pollen tube wall and a larger molecule in the stylar transmitting tract epidermis by acting directly in adhesion process or acting as a carrier of lipophilic compounds that, in turn, act as adhesion molecules. If the latter is true, bearing in mind the developmental context in which *GLTP1* is expressed, it is tempting to speculate that one possible role of GLTP1 development is to participate in directing and carrying lipophilic cell wall components during the growth of the ray flower corollas and carpels.

GEG participates in the regulation of cell and organ shape most likely by inhibiting cell elongation in the axial direction during late morphogenesis of corollas and carpels in gerbera. But in what cellular processes is GEG participating, in other words what cell biological role does GEG have? In light of the present data, three different hypotheses can be made: First, it could interfere with vesicle trafficking needed for example for cell wall synthesis/maintenance and thus guide the direction of organ growth. Second, it could act as an adhesion molecule between the cell membrane and cell wall or thirdly, be a cell wall component which, for example, by adhering to other molecules, preventing the sliding of cell wall matrix components, thus inhibiting cell elongation.

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