Molecular and Genetic Characterization of New MADS-box Genes in *Antirrhinum majus*

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To my family

Abbreviations

b-**gal**	β-galactosidase
3-AT	3-amino-1,2,4-triazole
A. majus	*Antirrhinum majus*
A. thaliana	*Arabidopsis thaliana*
A. tumefaciens	*Agrobacterium tumefaciens*
AGAMOUS	AG
ANR1	*Arabidopsis* NITRATE REGULATED 1
AP1	APETALA1
AP3	APETALA3
bp	base pair
CAL	CAULIFLOWER
CaMV	Cauliflower mosaic virus
cDNA	complementary deoxyribonucleic acid
CHO	CHORIPETALA
CO	CONSTANS
CTAB	N-Cetyl-N, N, N-trimethyl-ammonium bromide
DEF	DEFICIENS
DESP	DESPENTEADO
E. coli	*Escherichia coli*
EDTA	ethylene diamine tetraacetic acid
FAR	FARINELLI
FBP1	FLORAL BINDING PROTEIN 1
FBP11	FLORAL BINDING PROTEIN 11
FBP2	FLORAL BINDING PROTEIN 2
FBP3	FLORAL BINDING PROTEIN 3
FBP7	FLORAL BINDING PROTEIN 7
FIS	FISTULATA
FLC	FLOWERING LOCUS C
FUL	FRUITFULL
GLO	GLOBOSA
GP	GREEN PETAL
HEPES	4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid
kb	kilo base
Kn1	Knotted1
LFY	LEAFY
LUG	LEUNIG

MCM1	MINICHROMOSOME MAINTENANCE1
MEF2	myocyte enhancer factor 2
MOPS	3-(*N*-morpholino)-propanesulfonic acid
mRNA	messenger ribonucleic acid
MS	Murashige and Skoog
NAOAC	sodium acetate
NH4OAC	ammonium acetate
PEG	polyethyleneglycol
PFG	PETUNIA FLOWERING GENE
pfu	plaque forming units
PI	PISTILLATA
PLE	PLENA
PVP	Polyvinylpyrrolidone
RT-PCR	reverse transcription-polymerase chain reaction
SDS	sodium dodecyl sulfate
SEP1,2,3	SEPALLATA 1, 2, 3
SHP1	SHATTERPROOF 1
SHP2	SHATTERPROOF 2
SI1	SILKY 1
SOC1	SUPPRESSOR OF OVEREXPRESSION OF CO 1
SQUA	SQUAMOSA
SRF	serum response factor
STY	STYLOSA
SUP	SUPERMAN
SVP	SUPPRESSOR OF VEGETATIVE PHASE
Tam	transposon antirrhinum majus
TFL	TERMINAL FLOWER
TM3	TOMATO MADS-BOX GENE 3
TM5	TOMATO MADS-BOX GENE 5
ZMM2	*Zea mays* MADS 2

CONTENTS

INTRODUCTION *CHAPTER 1*

In the past decade a great breakthrough has been made in our understanding of flower development by studying flower homeotic mutants, mainly in the two model species *Antirrhinum majus* and *Arabidopsis thaliana*. The application of molecular biology to study floral homeotic mutants has identified a number of genes, which control developmental programs; most of them belong to the MADS-box gene family of transcription factors.

1.1. The MADS-box gene family

MADS-box transcription factors are named after the initials of the four originally identified members (Schwarz-Sommer et al., 1990). The yeast MINICHROMOSOME MAINTENANCE 1 (MCM1) protein regulates mating-type-specific gene expression (Herskowitz, 1989; Treisman and Ammerer, 1992), the *Arabidopsis* AGAMOUS (AG) (Yanofsky et al., 1990) and *Antirrhinum* DEFICIENS (DEF) proteins (Sommer et al., 1990) play regulatory roles in specifying the identity of floral organs, and the human serum response factor (SRF) is involved in the transcriptional regulation of the protooncogene c-*fos* (Treisman, 1986, 1987).

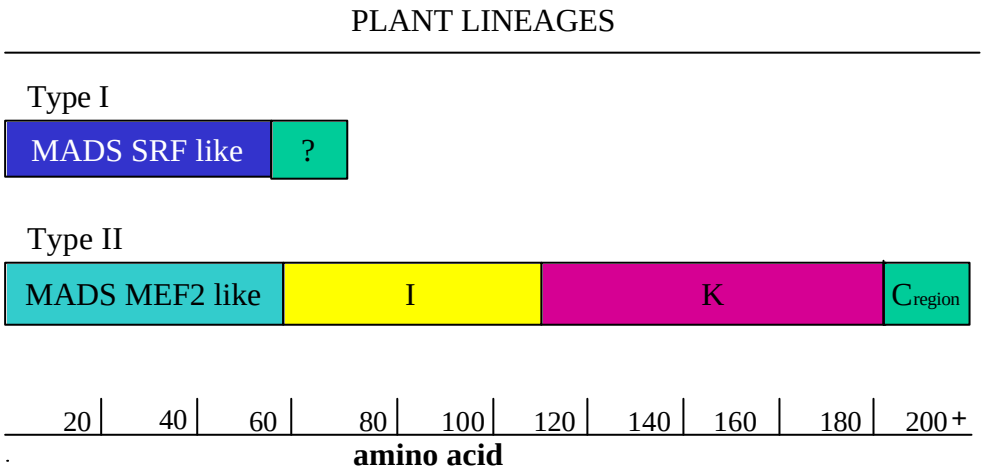

Figure 1-1. Schematic representation of the protein domains of plant type I (SRF-like) and type II (MEF2-like) MADS-domain proteins. The scale indicates the number of amino acids along the protein. The "?" in plant typeI-like proteins indicates that the C-terminus is not well defined yet and is of variable lengths (Alvarez-Buylla et al., 2000).

Phylogenetic analysis of the MADS-box gene family identified two MADS-box lineages named type I and type II in plants, animal and fungi (Alvarez-Buylla et al., 2000) (Figure 1-1). The two classes differ in the amino-acid consensus sequence of the MADS-box domain. TypeI MADS-box genes in plants resemble the animal serum response factor (*SRF*) in their MADS-box. They usually lack a K-domain (see below) and have not been characterized

functionally so far (Alvarez-Buylla et al., 2000; Svensson et al., 2000). Most plant MADS-box proteins fall into the type II category. Their MADS-box resembles the animal myocyte enhancer factor 2 (*MEF2*) gene (Alvarez-Buylla et al., 2000). In addition, plant MADS-box proteins are structurally related in that they are composed of the N-terminal M (MADS) domain, followed by the I (intervening), K (keratin like), and C (carboxyl-terminal) domains.

1.1.1 The modular structure of plant MADS-box proteins

By mutational and functional analysis it has been demonstrated that MADS-box proteins consist of a DNA-binding region, a region which serves as an interface for dimerization and interactions with other proteins, and sometimes contain a transcriptional activation domain. There is a considerable overlap between these functional domains and the M, I, K and C structural domains, although none of the functions can exclusively be assigned to just one single domain. Such modular organization is common to many eukaryotic transcription factors.

1.1.1.1 DNA-binding

DNA binding by plant type II MADS-box proteins to cis-acting promoter elements, named the CArG-box, is mediated by the MADS-domain, but also the K-box is believed to contribute to the binding specificity (see below). Studies to identify the minimal DNA-binding domain of the *Antirrhinum* MADS-box proteins SQUAMOSA (SQUA) and PLENA (PLE) demonstrated that the MADS- and I-domains are sufficient to permit sequence-specific DNA binding by the proteins (West et al., 1998). Similar results were obtained for the Arabidopsis MADS-box proteins APETALA1 (AP1), APETALA3 (AP3), PISTILLATA (PI), and AGAMOUS (AG). In the case of AP3 and PI the regions involved to form a protein-DNA complex are the MADS box, the entire I region and the first putative amphipathic helix of the K box, while for AP1 and AG only the MADS-box and part of the I region is needed (Riechmann et al., 1996a, b). For DNA binding the MADS-box proteins have to homo- and/or heterodimerize (see below). The differences in organization and partner specificity of the AP1, AG and AP3 and PI proteins support the idea that selective interactions achieve their functional specificity. Since the DNA-binding activities of the dimers (AP1-AP1, AP3-PI and AG-AG) are very similar, it is suggested that their biological specificity is achieved through selective interactions with additional transcription factors. This mechanism appears to be a common theme for MADS-box proteins of animals and fungi. DNA binding is often accompanied by transcription factor-induced DNA bending,

which is important in determining local promoter architecture and is thought to be a key determinant of their function, but the mechanism is still unclear (West and Sharrocks, 1999).

1.1.1.2 Protein-protein interactions: Dimerization and ternary complex formation

The partially conserved K-box of plant MADS-box proteins contains approximately 70 residues. (Ma et al., 1991). Secondary structure predictions reveal that the K-box has the potential to form amphipathic helices (Ma et al., 1991; Pnueli et al., 1991; Schwarz-Sommer et al., 1992). One possible function of the K-box is that it enables dimerization between sub-family members in a manner analogous to the leucine zipper motif (Davies and Schwarz-Sommer, 1994). The *Antirrhinum* MADS-box proteins DEF and GLOBOSA (GLO) have been shown to bind DNA as heterodimers *in vitro* (Schwarz-Sommer et al., 1992; Tröbner et al., 1992). Yeast two-hybrid experiments to determine the domains required for protein-protein interaction between DEF and GLO revealed that, although the MADS-box could be removed completely, deletions within the K-box resulted in the loss of interaction (Davies et al., 1996). *In vitro* DNA-binding studies with C-terminal deletion derivatives of DEF and GLO are in agreement with these observations (Tröbner et al., 1992; Zachgo et al., 1995). The involvement of the K-box in heterodimerization *in vivo* has also been demonstrated; An amino acid deletion within the K-box of a temperature-sensitive mutant of DEF, confers a modified phenotype, thus pointing to the importance of this domain in DEF function (Schwarz-Sommer et al., 1992). Furthermore, *in situ* immunolocalization assays revealed that DEF and GLO are only stable in the presence of the partner proteins, suggesting that heterodimerization contributes to their stability *in vivo* (Zachgo et al., 1995).

In addition to the K-box, at least part of the MADS-box is also involved in protein-protein interactions. A small structural alteration at the C-terminal end of the MADS-box of GLO impairs its function in the *glo*-confusa mutant *in vivo* (Zachgo et al., 1995). Similarly, *in vitro* studies with the Arabidopsis MADS-box proteins PI and AP3, the functional homologues of GLO and DEF, demonstrated that interactions between them are weakened by deletions in the K-box, and that removal of residues encompassing the MADS-box almost entirely abolishes their association (Goto and Meyerowitz, 1994).

In yeast, the SQUA, DEF and GLO proteins form ternary complexes via their C-termini, and in gel-shift assays the ternary complex shows enhanced DNA binding to consensus binding sites (the so-called CArG motifs) compared to DEF/GLO heterodimers or SQUA/SQUA homodimers (Egea-Cortines et al., 1999). Removal of the C-terminal domain from any of

these three *Antirrhinum* MADS proteins prevents both ternary association in yeast and the formation of higher order DNA-binding complexes in gel shift assays. Thus, the C-domain is required for the ternary complex formation between DEF, GLO and SQUA (Egea-Cortines et al., 1999). Evidence that the C-terminal domain is necessary for the function *in vivo* comes from experiments that ectopically express truncated versions of MADS proteins; such truncations result either in loss of activity or a dominant-negative phenotype (Krizek and Meyerowitz 1996; Mizukami et al., 1996).

1.1.1.3 Transcriptional activation

In yeast experiments, some of the plant MADS-box factors can activate transcription that is dependent on the presence of the C-domain. The amino acid sequence within the C-domain is very divergent with the exception of some short sequence motifs within the region and a few amino acids at the extreme end, which are often conserved between MADS-box factors performing similar roles in different species. Although obvious activation domains are lacking, the C-domains of some MADS-box proteins can probably be involved in activation or repression of transcription. It has been demonstrated in yeast that the C-terminus of DEFH49 from Antirrhinum contains an activation domain (Davies et al., 1996). Similarly, analyses of the C-terminal domains of AP1 from *Arabidopsis* and its homologues *RsMADS1* from *Raphanus sativus* (radish), *NsMADS2* from *Nicotiana sylvestris* (long-day tobacco) as well as *NtMADS5* from *Nicotiana tabacum* (day neutral tobacco) showed a transcriptional activation function of the C-domain (Cho et al., 1999).

1.1.2 Evolution of the MADS-box gene family

Different plant MADS-box factors share the same organization of regions and the highly conserved amino acid sequence of the MADS-domain. This indicates that these genes were derived from a common evolutionary ancestor. After the isolation of the first plant MADS-box genes from plant *DEF* and *AG* (Sommer et al., 1990; Yanofsky et al., 1990), a large number of MADS-box genes have been sequentially identified in different species in angiosperms, and the orthologues of floral homeotic MADS-box genes have also been found in gymnosperm (Munster et al., 1997; Becker et al., 2000). The members of this multigene family are grouped by sequence similarity in distinct subfamilies or monophyletic gene clades in the phylogenetic trees (Purugganan et al., 1995; Theissen et al., 1996). Members of a subfamily tend to have related expression patterns and functions (Theissen et al., 1996). This might reflect that the primary structure and the regulatory function of these genes were

tightly linked to each other during evolution. Gene duplication and sequence diversifications were the most common mechanisms used for the creation of new genes throughout the evolution of the MADS-box gene family (Theissen et al., 1996; Purugganan, 1998).

1.2 Diverse functions of MADS-box genes in plants

1.2.1 Control of flowering time by MADS-box genes

In plants, the functions of MADS-box genes are best understood during reproductive development, including the control of flowering time. Flowering time is influenced by both environmental conditions, which include day length, temperature, light quality, nutrient deprivation, and developmental factors associated with the age of the plant (Koornneef et al., 1998). A large number of flowering mutants are available in *Arabidopsis* and have been classified into early mutants which advance flowering in comparison to the wild type, and late mutants that delay flowering time. Genetic analyses of late-flowering mutants identified more than 20 genes. The mutants involved in the control of this process were physiologically classified and fall into three genetic pathways on the basis of their responsiveness to environmental factors (Simpson et al., 1999; Devlin and Kay, 2000; Samach and Coupland, 2000). The first class belongs to the autonomous pathway, which promotes the transition from vegetative to reproductive development under both long-day and short-day conditions. MADS-box genes like *FLOWERING LOCUS C* (*FLC*) and *SHORT VEGETATIVE PHASE* (*SVP*) belong to this pathway. Both of them negatively regulate the transition, but *FLC* is more central for the pathway (Michaels and Amasino, 1999; Hartmann et al., 2000). The second pathway is the photoperiodic pathway (also called the long-day pathway) which promotes flowering only under long-day conditions but has no effect under short days. *CONSTANS* (*CO*), a zinc-finger protein is involved in this pathway (Putterill et al., 1995). The day-length independent pathway (also called the gibberellin pathway) stimulates flowering by the plant hormone gibberellin. It has been shown that the MADS-box gene *SUPPRESSOR OF OVEREXPRESSION OF CO 1* (*SOC1*) can integrate signals from all three pathways (Lee et al., 2000; Onouchi et al., 2000; Samach et al., 2000) and that it is a direct target of *CO* (Onouchi et al., 2000; Samach et al., 2000).

Compared to late-flowering genes, less is known about early-flowering genes. *TERMINAL FLOWER* (*TFL*) controls both flowering time and the identity of the shoot meristem (Shannon and Meekss-Wagner, 1991; Alvarez et al., 1992). Therefore, *TFL* provides a link

between the control of flowering time and flower initiation. How MADS-box genes like *AP1, CAULIFLOWER* (*CAL*), *FRUITFULL* (*FUL*) and *SVP*, which also control flowering time, are integrated into the current framework is still unclear (Mandel et al., 1992; Kempin et al., 1995; Mandel and Yanofsky 1995; Ferrendiz et al., 2000; Hartmann et al., 2000).

1.2.2 Control of floral meristem identity

In flowering plants, the transition from vegetative to reproductive growth is a critical developmental process, which is marked by a number of changes in the shoot apex at the molecular, physiological and morphological level. The switch from vegetative to reproductive development leads to the production of flowers instead of leaves or shoots and requires the activity of floral meristem identity genes whose expression is upregulated in developing floral primordia during the transition. Mutant plants of such genes develop shoots or shoot-like structures in place of flowers. These genes include *SQUA* in *Antirrhinum, PETUNIA FLOWERING GENE* (*PFG*) and three closely related genes from *Arabidopsis AP1, CAL and FUL,* as well as the non-MADS-box gene *LFY*. Mutation in the *SQUA* gene results in the development of bract-forming shoots at positions where normally flowers would develop (Huijser et al., 1992), and inhibition of *PFG* expression in transgenic plants, using a cosuppression strategy, results in a unique phenotype without flowers (Immink et al., 1999). The *AP1* and *CAL* genes have overlapping functions in promoting flower meristem identity and *ap1 cal* double mutants have a massive proliferation of a shoot-like meristem in positions normally occupied by a single flower (Bowman et al., 1993). This phenotype is further enhanced by mutations in *FUL,* such that *ful ap1 cal* triple mutants never flower under standard growth conditions, and continuously elaborate leafy shoots in place of flowers (Ferrendiz et al., 2000). This observation indicates that these three genes act together to control meristem identity. The failure to flower in the triple mutant is due to loss of *LFY* upregulation, because introducing a transgene that constitutively expresses *LFY* into the *ful ap1 cal* background restores flowering (Ferrendiz et al., 2000).

1.2.3 Control of floral organ identity

Typical flowers of eudicotyledonous plants are composed of four different types of organs, which develop sequentially from the outside to the inside on the flanks of the floral meristem. Each organ type is arranged in a concentric ring or whorl, numbered one to four from the outermost to the innermost. In whorl 1 sepals develop, in whorl 2 petals, which together constitute the perianth. The reproductive organs, stamens and carpels, constitute whorl 3 and

4, respectively. After the floral meristem is determined, the organ identity genes are activated to control downstream genes that are characteristic of the cells of each organ type. Organ identity genes are homeotic selectors; mutations in such genes cause the transformation of one organ type into another that normally does not develop in that whorl.

1.2.3.1 Genetic control of floral organ identity: The ABC model

Genetic studies in *Antirrhinum* and *Arabidopsis* using floral homeotic mutants led to a simple model (Coen and Meyerowitz, 1991) that proposes three classes of genes named A, B, and C to be expressed in adjacent, overlapping whorls of a flower. The A function is expressed in whorls 1 and 2, the B function in whorls 2 and 3, and the C function in whorls 3 and 4. Expression of the A function alone specifies sepal development, co-expression of the A and B functions or the B and C functions determines petal or stamen development, respectively, and expression of the C function alone results in carpel development. Loss of the A function causes transformation of first whorl sepals into carpelloid leaves in weak A-function mutants, and second whorl petals become stamenoid (Gustafson-Brown et al., 1994). In B loss-of-function mutants, sepals and carpels replace petals and stamens, respectively (Sommer et al., 1990; Jack et al., 1992; Tröbner et al., 1992; Goto and Meyerowitz, 1994). In C function mutants, petals develop in whorl 3 instead of stamens, and new flowers grow inside whorl 3 *in Arabidopsis* and inside whorl 4 *in Antirrhinum* (Yanofsky et al., 1990; Bradley et al., 1993; Davies et al., 1999). A and C functions are mutually antagonistic; thus, the A function excludes the C function from whorls 1 and 2 (the so-called cadastral function) and the C function excludes the A function from whorls 3 and 4 (Bowman et al., 1989; Bowman et al., 1991) (Figure 1-2A).

Double and triple mutant analyses revealed that, interestingly, the B function alone can control organ identity: in *Arabidopsis* AC double mutants whorls 1 and 4 have leaflike organs and whorls 2 and 3 have intermediate organs between petals and stamens (Bowman et al., 1991).

With the isolation of new MADS-box genes specifying ovule development from *petunia hybrida*, the ABC model was extended to include the D function (Angenent et al., 1995; Colombo et al., 1995; Figure 1-2B). There are two D function MADS-box factors in *petunia*, FLORAL BINDING PROTEIN7 (FBP7) and FBP11. Ectopic expression analyses of these two-D function genes revealed that they induce the formation of ovule-like structures on the perianth organs of transgenic flowers. Therefore, they have been considered as a new class of

master control genes specifying ovule development in *petunia*. Over the past decade, the ABC model of flower development has been widely appreciated in a large range of angiosperm species.

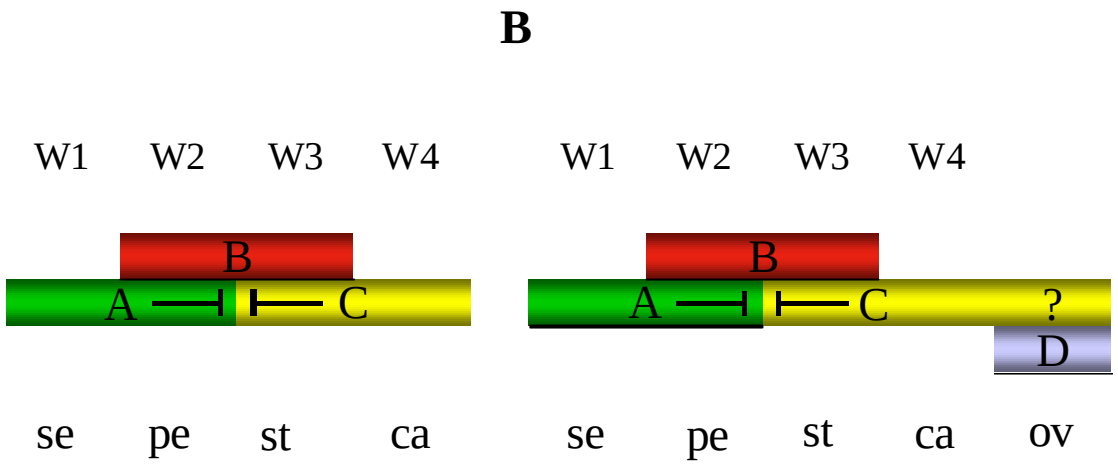

Figure 1-2. Molecular models of floral organ identity. (A) Most dicot flowers consist of four floral organs in concentric whorls: sepals (se) in the outmost whorl 1 (W1), petals (pe) in whorl 2 (W2), stamens (st) in whorl 3 (W3) and carpels (ca) in the center of whorl 4 (W4). The ABC model illuminates that three classes of homeotic genes function in a combinatorial manner to specify four floral organ identities and their activities are restricted to two adjacent whorls of the flower. In addition, the A- and C-class genes mutually repress the other's expression in their respective domains as shown in barred lines. (B) The ABC model is extended to the ABCD model by the addition of the D-function gene. The D function specifies ovule identity. Whether the C-function gene is involved in ovule development is not clear and is indicated by a question mark in whorl 4.

1.2.3.2 The ABC model at the molecular level

In *Arabidopsis* all representative A, B or C genes have been cloned. The *AP1* and *AP2* genes have A-class function as well as being meristem identity genes. *AP1* is a MADS-box gene, whereas AP2 is the founder of a novel gene family called AP2/EREBP-like genes (Riechmann and Meyerowitz, 1998). When floral organs are initiated, *AP*1 is expressed only in whorls 1 and 2. The *Antirrhinum AP*1 orthologue is SQUA, whose mutants, however, have a different phenotype; flowers are replaced by inflorescence shoots. On the basis of this homeotic transformation SQUA is required for the control of floral meristem identity. So far no effect of *SQUA* expression on the specification of sepal and petal organ identity has been demonstrated although subsequent to organ initiation *SQUA*, like *AP1*, is mainly expressed in whorls 1 and 2. Therefore, at present, *Arabidopsis* is the only species in which A function genes with the dual cadastral and organ identity functions have been identified.

The B function genes *DEF* and *GLO* from *Antirrhinum* and *AP3* and *PI* from *Arabidopsis* are expressed mainly in the second and third whorls and show similar mutant phenotypes. In

petunia there are three genes showing the B function expression pattern. The *FLORAL BINDING PROTEIN 1* (*FBP1*) gene is most closely related to the *Antirrhinum GLO* and Arabidopsis *PI* gene, its inactivation causes the same homeotic transformation as *GLO* and *PI* do, in that sepals replace petals and carpels replace stamens (Angenent et al., 1993). The *GREEN PETAL* (*GP*) gene, the homologue of *DEF* and *AP3*, is expressed in the petal and stamen primordia, but the null mutant shows a homeotic effect in only one whorl; the petals are converted to sepals and the third whorl stamens are not affected (van der Krol et al., 1993). The third gene, *FBP3*, is closely related to *GLO* and *PI* and also expressed in developing petals and stamens (van der Krol et al., 1993). It is assumed that *FBP3* might specify stamen development.

There is evidence that the basic mechanisms of flower development are conserved between grasses and eudicots (Goto et al., 2001; Ng and Yanofsky 2001), although the flower structures of monocot grass plants and eudicot flowers are highly divergent. In male flowers of the maize mutant *silky1* (*si1*), stamens are replaced by feminized bract-like structures and the lodicules are replaced by bracts. In the female flowers the lodicules show similar transformations as in the male flowers, and extra carpels replace stamens. *Si1*, the maize orthologue of *AP3*, is expressed in the lodicules and stamens (Ambrose et al., 2000).

The rice gene *OsMADS4* is the putative orthologue of the Arabidopsis B function gene *PI* (Kang et al., 1998). Flowers expressing antisense *OsMADS4* display alterations of the second and third whorls in transgenic rice plants. The second-whorl lodicules, which are equivalent to the petals of dicot plants in grasses, were altered into palea/lemma-like organs and the third whorl stamens to carpel-like organs (Kang et al., 1998).

The C function genes specify stamen and carpel development and are also involved in the control of determinacy. In *Antirrhinum* there are two closely related C-function genes, *PLE* and *FARINELLI* (*FAR*). The expression patterns of *PLE* and *FAR* are similar, but the phenotypes of their mutants are very different. In *ple* mutants, petals replace stamens and inside whorl 4 sepaloid/carpeloid/petaloid organs develop. In contrast, *far* mutants show only partial defects in stamens and no defect in carpels (Davies et al., 1999). Double mutant analyses revealed that the two genes synergistically control flower determinacy. In maize, two closely related *AG*-like MADS-box genes, *ZAG1* and *Zea mays* MADS 2 (*ZMM2*) have been identified, which are expressed during ovule and carpel development (Schmitz et al., 1993; Mena et al., 1996). *Zag1* mutant flowers show loss of determinacy but no defects in

organ identity. It has been speculated that *ZMM2* might be responsible for the organ identity control. With the completion of the sequence of the *Arabidopsis* genome (The *Arabidopsis* Genome Initiative), it is now known that there are more than 80 MADS-box genes in *Arabidopsis* (Riechmann, 2000). The genome sequence reveals many closely related genes, which might have a common redundant function, and which is not obvious by analyzing their mutants. This has been shown to be true for the three floral *SEP* MADS-box genes, which are similar in sequence and exhibit similar temporal expression patterns early during flower development (Flanagan and Ma, 1994; Savidge et al., 1995; Mandel and Yanofsky, 1998). Neither of the *sep* single mutants nor combinations of double mutants exhibit a dramatic developmental phenotype. However, the *sep1 sep2 sep3* triple mutant exhibits a phenotype that is similar to BC double mutants *pi ag* or *ap3 ag*. This result suggests that all three *SEP* genes together are necessary for the development of petals, stamens and carpels and that they define a new class of organ identity function that is required for the activities of the B- and C-function genes (Pelaz et al., 2000). The *SEP* function might be conserved in distantly related eudicots. *FLORAL BINDING PROTEIN 2* (*FBP2*) in petunia and *TOMATO MADS-BOX GENE 5* (*TM5*) in tomato are SEP orthologues (Angenent et al., 1994; Pnueli et al., 1994). Both of them are expressed in petals, stamens and carpels. Cosuppression and antisense experiments using *FBP2* and *TM5* result in transgenic plants with flowers defective in the three inner whorls. In both cases petals are converted into sepal- or leaf-like organs, and additional whorls of organs or new flowers can grow in the center of the flowers. Orthologues of *SEP* genes have been identified also in the monocot species rice (Kang and An, 1997) and in the non-flowering gymnosperms Monterey pine (Mouradov et al., 1998).

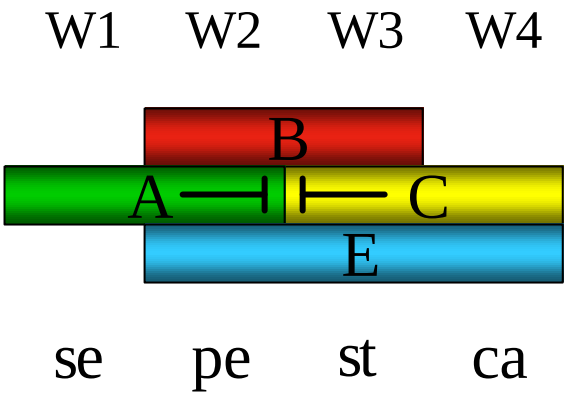

Figure 1-3. The quartet model of floral organ identity. The abbreviation used in this figure is the same as in figure 1-2A. The A, B, and C genes function as the same as in the ABC model (see Figure 1-2A). SEP1, and/or SEP2, and/or SEP3 provide the E function, which is required for the determination of petal, stamen and carpel identity. Barred lines represent antagonistic interactions.

Based on multimeric complex formation between the A-, B-, and C-function proteins (1.1.1.2) and the SEP proteins (Goto et al., 2001; Honma and Goto, 2001; Jack, 2001), and on mutant phenotypes combined with studies with transgenic plants in *Arabidopsis* mentioned above, the ABC model was further revised to the quartet model of flower organ identity (Theissen and Saedler, 2001). In this model the A-function, in combination with unknown components, specifies sepals in whorl 1; A-, B-function, and *SEP* genes together specify petals in whorl 2; B-, C-function, and *SEP* genes together determine stamen identity in whorl 3; and C-function and *SEP* genes together determine carpels in whorl 4 (Figure 1-3).

1.2.4 Functions of MADS-box genes beyond the flower

Most studies on MADS-box genes unraveled their functions in flower development, but it is known that they also play roles in vegetative development of the plant. The *NMH7* gene in alfalfa is expressed in infected cells of root nodules, and it is postulated that it might be involved in the signal transduction pathway initiated by the bacterial symbiont *Rhizobium meliloti* (Heard and Dunn, 1995). The *Arabidopsis NITRATE REGULATED 1* (*ANR1*) gene controls root growth in response to nitrate (Zhang and Forde, 1998); the tomato *JOINTLESS* gene is a key regulator for abscission zone development (Mao et al., 2000); and *AGL16* is assumed to play regulatory roles in trichomes and guard cells (Alvarez-Buylla et al., 2000).

Some MADS-box genes are involved in the specification of cell fates in the fruit. *MdMADS4* from the apple cultivar *Fuji* (Malus x domestica Borkh) is highly expressed in the vascular bundles in the floral tube and the carpellary vascular bundles in the fruit at early developmental stages, so it has been suggested that it may function in fruit development (Sung et al., 2000). In *Arabidopsis*, three MADS-box genes *FRUITFULL* (*FUL*), *SHATTERPROOF 1* (*SHP1*) and *SHATTERPROOF 2* (*SHP2*) determine cell fate in the development of the fruit. *SHP1* and *2* are closely related, functionally redundant, and involved in the differentiation of the dehiscence zone (Liljegren et al., 2000). Differentiation of the valves requires the activity of *FUL*, which negatively regulates *SHP1* and *2* expression (Ferrandiz et al., 2000). It has been reported that DEFH28, the orthologue of FUL in Antirrhinum, may regulate fruit maturation (Muller et al., 2001).

1.3 Regulation of MADS-box gene expression

Plant MADS-box genes are often expressed at the time and place where their products are required, indicating a sophisticated transcriptional control of their expression (Soltis et al.,

2002). Two classes of upstream regulators have been identified to regulate the expression of the homeotic genes: so called floral meristem identity genes and cadastral genes. Double mutants of the floral meristem identity genes *LEAFY* (*LFY*) and *AP1* in *Arabidopsis* exhibit an extreme conversion of flowers into shoots in which the organ identity gene *AG* is no longer activated in its normal pattern (Weigel and Meyerowitz, 1994). Cadastral genes appear to demarcate the initial expression domains of organ identity genes. *SUPERMAN* (*SUP*) and *LEUNIG* (*LUG*) are two cadastral genes identified in *Arabidopsis* (Bowman et al., 1992; Liu and Meyerowitz, 1995; Sakai et al., 1995; Conner and Liu, 2000). Single and double mutant analysis demonstrated that *SUP* is involved in the repression of the class B genes *AP3* and *PI* in whorl 4, whereas *LUG* is required to repress the expression of the class C gene *AG* in whorls 1 and 2. It is still unclear how these cadastral genes establish the spatial patterns. A similar mechanism has also been reported in *Antirrhinum*, where *STYLOSA* (*STY*) and *FISTULATA* (*FIS*) together prevent the G-function gene *PLE* to expand towards the perianth (Motteet al., 1998). Similarly, the *CHORIPETALA (CHO* and *DESPENTEADO* (*DESP*)) genes negatively regulate the expression of class B and C genes in whorl 1 (Wilkinson et al., 2000).

Another control mechanism involves autoregulation. In *Antirrhinum*, the heterodimer formed between DEF and GLO can bind *in vitro* to cognate sites (the CArG motif) present in the promoters of both genes and upregulates their transcription (Schwarz-Sommer et al., 1992, Tröbner et al., 1992; Zachgo et al., 1995). A similar regulation was also observed for the DEF and GLO orthologues, AP3 and PI in Arabidopsis (Goto and Meyerowitz, 1994; Jack et al. 1994).

Expression of some MADS-box genes is induced not only by developmental signals, but also in response to environmental stimuli. For instance, in *Antirrhinum* the *DEFH125* gene is induced by pollination within the upper part of the transmitting tissue of carpels (Zachgo et al., 1997), in alfalfa the *NMH7* gene is induced in roots after *Rhizobium* infection, and in *Arabidopsis* the *ANR1* gene is induced in roots by NO_3^- rich soil patches, leading to an altered root architecture (Heard and Dunn, 1995; Zhang and Forde, 1998). Interestingly, all these genes belong to the same subfamily of MADS-box factors. Another possible mechanism for regulating MADS-box factors involves subcellular localization. It has been observed in onion cells that AP3 and PI have to be coexpressed to be localized inside the nucleus (McGonigle et al., 1996).

1.4 The MADS-box protein network

Many biological functions involve the interaction between proteins and, as in many other instances, screens utilizing the yeast two-hybrid system provided a powerful tool to search for partners in MADS-box protein complexes. Using the GLO protein as a bait only DEF could be identified as an interaction partner (Davies et al., 1996). In contrast, when similar experiments were performed with PLE, a number of interacting partners were isolated, such as SQUA, DEFH49, DEFH72 and DEFH200 (Davies et al., 1996). The same has been observed in Arabidopsis using the PLE homologue AG as a bait, leading to the identification of SEP1, SEP2 and SEP3 as interacting proteins (Fan et al., 1997). All of these partners are members of the MADS-box family.

As mentioned before (1.1.1.2), MADS proteins in plants might associate in complexes larger than dimers (Egea-Cortines and Davies, 2000). First evidence that these complexes are functional came from *in vitro* DNA-binding assays. A DNA probe containing two CArG box sequences was bound stronger in the presence of all three MADS-box proteins, SQUA, DEF, and GLO, compared to the binding by the homodimer of SQUA or the heterodimer of DEF and GLO (Egea-Cortines et al., 1999). Similar interactions between AP3, PI and AP1 and between AP3, PI and SEP3 were confirmed by co-immunoprecipitation experiments (Honma and Goto, 2001). The *in vivo* relevance of the finding that MADS-box proteins can form higher order complexes has been corroborated by genetic studies with the SEP genes (1.2.3.2). These studies pointed to the crucial role of ternary complex formation between the SEP proteins and class A, B and C proteins in the control of floral organ identity (Goto et al., 2001). It is likely, that such higher order complexes can be formed between several other MADS-box proteins, and that these additional combinations further contribute to the diversity of control events governed by MADS-box proteins in plants.

In contrast to animal and yeast MADS-box factors, there is currently no well-characterized interaction between a plant MADS-box protein and a non-MADS transcription factor (Egea-Cortines and Davies, 2000), although some evidence implies that the plant MADS-box factors might have this feature (Davies and Schwarz-Sommer, 1994).

1.5 Objectives of this dissertation

So far 24 MADS-box genes have been isolated from *Antirrhinum* (see table 1-1), but, because of extensive gene duplications of key regulatory molecules (Martienssen and Irish, 1999),

more family members are expected to exist. In order to better understand the role of the MADS-box gene family during different aspects of plant development, the isolation and characterization of new family members was the main objective of this thesis.

MADS-box transcription factors form intricate networks through protein-protein interactions. Therefore, novel genes were used as a bait in the yeast two-hybrid system to identify their interactions with known members of the MADS-box family, and possibly, to isolate additional, formerly unidentified family members.

Table 1-1. MADS-box genes in *A. majus*

Gene	Expression	Function	Reference
SQUA	early inf. and flr.	A function	Huijser et al., 1992
DEF	flr. 2,3	B function	Sommer et al., 1990
GLO	flr. 2,3	B function	Tröbner et al., 1992
PLE	flr. 3,4	C function	Bradley et al., 1993
FAR	flr. 3,4	C function	Davies et al., 1999
DEFH2	flr. 1	unknown	
DEFH9	flr. 4	unknown	
DEFH11	leaves and bracts	unknown	
DEFH21	flr. 4	unknown	Becker et al., 2002
DEFH24	vegetative and flr. 2,3,and 4	unknown	
DEFH28	inf., carpel wall	unknown	Müller et al., 2001
DEFH49	flr. late	unknown	Davies et al., 1996
DEFH52	flr. 1,2,4	unknown	
DEFH57	unknown	unknown	
DEFH70	unknown	unknown	
DEFH72	intermediate flr.	unknown	Davies et al., 1996
DEFH76	unknown	unknown	
DEFH76B	unknown	unknown	
DEFH83	unknown	unknown	
DEFH84	intermediate flr.	unknown	
DEFH101	capsule, roots and flr. 1	unknown	
DEFH102	embryo	unknown	
DEFH125	pollen and stamens	unknown	Zachgo et al., 1997
DEFH200	intermediate flr.	unknown	Davies et al., 1996

* inf. = inflorescence; **flr. = flower; 1-4 = floral whorls

MATERIAL AND METHODS

CHAPTER 2

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2.1 Chemicals, enzymes and oligonucleotides

Chemicals used for these experiments were purchased from the following companies: Life Technology Pharmacia (Freiburg), Sigma (Deisenhofen), Merck (Darmstadt), Biomol (Hamburg), Fluka (Neu-Ulm) and Promega (Madison). Nylon membranes were obtained from Amersham (Braunschweig), Radioisotopes [α^{32}P]-dCTP (10 µCi/µl), [γ^{32}P]-ATP (10 µCi/µl) and [^{35}S]-Methionine (50 µCi/µl) from Amersham Buchler (Braunschweig).

Enzymes were purchased from Roche (Mannheim), Biolabs (England), Life Technology (Freiburg), MBI Fermentas (St. Leon-Rot), Pharmacia (Freiburg) and Sigma (Deisenhofen). 10 x buffers were supplied together with the corresponding enzymes.

Oligonucleotides were synthesized at Life Technology (Freiburg) and Metabion (Martinsried).

2.2 Plant materials

Antirrhinum wild-type lines Si50 and 165E, mutants *impressa*, *sippe2249*, *marmorea*, *mat hero lat elo* and *sippe ragusa*, and Tam elements mutagenized plants were grown at 18-25°C (16 hr light/8 hr dark) in greenhouse.

Arabidopsis wild type Columbia and mutant *soc1* were also grown in the greenhouse in long-day (16 hr light/8 hr dark) and short-day (10 hr light/14 hr dark) conditions.

2.3 Bacterial strains

Escherichia coli

DH10B F¯, *mcr*AΔ (*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15, Δ*lac*X74, *deo*R, *rec*A1, *end*A1, *ara*D139, Δ (*ara, leu*)7607, *gal*U, *gal*K, λ¯ *rps*1, *nup*G.

K803 F¯, el4¯(McrA¯), *lac*Y1, or. Δ (*lac*)6, *sup*E44, *gal*K2, *gal*T22, *rfb*D1, *mcr*V1, *hsd*S3, (r_k¯, m_k¯).

Agrobacterium tumefaciens

GV3101 (Van Larabeke et al., 1974)

2.4 Yeast strains

SFY526 *MATa, ura3-52, his3-200 ,ade2-101, lys2-801, trp 1-901, leu2-3, 112, canr, gal542, gal80-538, URA3::GAL 1UAS-GAL 1TATA-LacZ*

Y190 *MATa, ura3-52, his3-200, ade2-101, lys2-801, trp 1-901, leu2-3, 112, gal4D, gal80D, cyh^{r}2, LYS2::GAL 1$_{UAS}$-HIS3$_{TATA}$3, MEL1, URA3::GAL 1$_{UAS}$-GAL 1$_{TATA}$-LacZ*

2.5 Cloning vectors

pBluescript KS (+)	(Stratagene)
pBluescript SK (+)	(Stratagene)
pGEX-5X-1	(Pharmacia Biotech)
pGAD424	(Clontech)
pGBT9	(Clontech)
pH35S XS	(Yephremov A. unpublished data)
pB35S XS	(Yephremov A. unpublished data)
pGEM-T	(Promega)
pPCV702	(Koncz et al., 1994)

2.6 Southern Blot

2.6.1 Plant genomic DNA extraction

Genomic DNA was extracted from *Antirrhinum* plants as following: frozen leaves (1-10 g) were ground in a mortar to fine powder, then poured into an appropriate tube containing CTAB extraction buffer (5 ml buffer/gram material), incubated at 60°C for 30 min with occasional stirring, filtered through Miracloth squeezing thoroughly; the lysate was extracted with one volume of chloroform, shaked vigorously, and centrifuged 15 min at 5,000 rpm. The supernatant was transferred to a new falcon tube, precipitated with 0.8 volume isopropanol, kept at RT for 5 min, then spun at 5,000 rpm for 15-20 min. The pellet was briefly washed with 70% EtOH, air dried, resuspended in 500 µl TE/RNase, and incubated at 37°C in a waterbath for 30-60 min. After that, the Qiagen column was used to repurify DNA (according to the PLANT MINIPREP KIT protocol). Extracted DNA was quantified by both spectrophotometer measurement and by intensity comparison on an ethidium bromide-stained agarose gel with a DNA molecular weight standard.

CTAB extraction buffer:		**TE buffer:**	
Tris/HCl pH 8	100 mM	Tris/HCl pH 8	10 mM
NaCl	1.4 M	EDTA	0.1 mM
EDTA	20 mM		
CTAB	2%	**TE/RNase:**	
add 0.2% β-mercaptoethanol before use		20 ug/ml RNase A in TE	

2.6.2 DNA digestion, separation and transfer to membranes

Purified genomic DNA was digested with AluI, AvaII, BamHI, EcoRI, EcoRV, HindIII and XbaI at 37°C in a waterbath overnight; the resulting genomic DNA fragments were separated on 0.6% agarose gels. After electrophoresis, the agarose gel was soaked in denaturing solution (1.5 M NaCl, 0.5 M NaOH) for 30 min; then the DNA fragments from the gel were transferred to a nylon membrane. The transferred DNA was immobilized by UV irradiation (Stratagene UV crosslinker, 120 mJ).

2.6.3 Radioactive labelling of probes

Random primer labelling

The templates used as probes were prepared by PCR amplification from DEFH7 and DEFH68 cDNA clones with gene-specific primers in non-conserved regions. Primer pairs 5'–TCA GGA AAG GCA TAT CAG TAT GCA AG-3' and 5'–ATG GTT GTC CTT GAA ACA GGT CCA-3' for DEFH7 and 5'–CCA GCA TTG AAC TTA CAG AAA GCG-3' and 5'–ATT TCA TGC TAG TTT CCA AGC C-3' for DEFH68 were used to amplify 467 bp and 270 bp downstream of the MADS-domain regions, respectively.

Labelling reaction

A mixture constituted by 3 µl of 10 x oligo mix, 3 µl [α^{32}P] dCTP (10 µCi/µl), 1.5 µl of Klenow polymerase (2 U/µl) in a final volume of 20 µl was added to 25-40ng of template DNA in volume of 10 µl previously denatured for 10 min at 100°C.

The resulting mixture was incubated at room temperature for 90 min and after that the reaction was stopped by addition of 2 µl of 0.5M EDTA. After addition of 2 µg herring sperm DNA and H_2O to a final volume of 50 µl, the probe was precipitated with 4M NH_4OAc (pH 6.0) and 200 µl ethanol for 30 min at room temperature, centrifuged 30 min at 14,000 rpm. at 20°C and briefly washed with 70% ethanol. The pellet was dried and dissolved in 300 µl of TE buffer.

10 X Oligo mix

dATP, dGTP, dTTP	0.2 mM
$(dN)_6$	4.0 mg/ml
HEPES, pH 6.6	2.0 M
Tris-HCl, pH 8.0	0.4 M
$MgCl_2$	0.074 mM
β-Mercaptoethanol	0.7% (v/v)

2.6.4 Hybridization

Pre-hybridization was performed for at least 20 min in the hybridization solution supplemented with 100 µg/ml herring sperm DNA at 68°C with gentle shaking; after adding the denatured probe, the hybridization was carried out overnight under the same condition. After hybridization the membranes were washed twice (15 min each time in the same condition mentioned above) with a solution containing 2 x SSPE and 0.1%SDS and exposed to Kodak X-ray films (X-omat AR) at –80°C in 3MM intensifying screens.

Hybridization solution	
SSPE	3x
SDS	0.1%
PVP	0.02%
Ficoll	0.02%

2.7 Northern Blot

2.7.1 Plant total RNA extraction

0.6 g of each *Antirrhinum* tissue was used to isolate total RNA, according to the protocol provided by Qiagen (THE QIAGENOLOGIST). Isolated total RNA was dissolved in 2 ml elution buffer (components listed in paragraph 2.7.2). The concentration of each total RNA sample was measured spectrophotometrically according to the following equation:

$[RNA]\ (\mu g/\mu l) = A_{260} \times 40/Vm$

where A_{260} is the absorbance at 260 nm and Vm is the volume of sample used for measurement in µl

2.7.2 Plant poly(A)$^+$ RNA extraction

After incubation at 65°C for 3 min, the total RNA was gently mixed with one volume of Dynabeads Oligo $(dT)_{25}$ (DEUTSCHE DYNAL GmbH), which was previously regenerated according to the supplier's instructions and resuspended in 2 x binding buffer. After 5-10 min incubation at room temperature, the mixture was placed on a magnet for at least 30 sec until the solution became clear and the supernatant was discarded; the Dynabeads were then washed twice by adding washing buffer, vortexing, placing the mixture on the magnet and discarding the supernatant; finally the poly(A)$^+$ RNA was eluted by addition of 700 µl of elution buffer, followed by vortexing, heating at 65°C for 2 min, and collection of the supernatant in a new tube; the supernatant was then precipitated with one volume of

isopropanol, washed with 80% ethanol, dried briefly on the bench and dissolved in TE buffer. The amount of poly(A)$^+$ RNA extracted from each sample was detected spectrophotometrically according to the equation in paragraph 2.7.1.

2 X Binding Buffer		**Washing Buffer**		**Elution Buffer**	
Tris-HCl	20 mM	Tris-HCl	10 mM	EDTA pH 7.5	2 mM
EDTA	2 mM	EDTA	1 mM		
LiCl	1 M	LiCl	0.15 M		
Final pH = 7.5		Final pH = 7.5			

2.7.3 RNA separation and transfer to membranes

2 µg poly(A)$^+$ RNA were mixed with 10 µl sample buffer and 1 µl loading buffer, denatured at 60°C for 10 min, and separated on a vertical denaturing agarose gel with 4 mm thick spacers and 0.5 cm slots. The electrophoretic separation was performed at 60 V for about 5 min, then at 50 V until the bromophenolblue marker had migrated about 9 cm. After electrophoresis, the gel was soaked for 5 min in water, denatured for 40 min in 50 mM NaOH/10 mM NaCl, neutralized for 25 min in 0.1 M Tris pH7.5 and equilibrated for 20 min in 20 x SSC or SSPE.

After overnight transfer in 20 x SSC or SSPE, the resulting filter was dried at 80°C for 30 min.

10 X Gel buffer		**RNA denaturing gel**	
MOPS	200 mM	Agarose	1.68 g
NaOAc	50 mM	10x gel buffer	14 ml
EDTA	10 mM	H_2O	98 ml
adjust pH to 7.0 with NaOH; store at 4°C in the dark.		dissolve in microwave oven, cool down to 60°C, add 28 ml formaldehyde 35%.	

Sample buffer		**RNA loading buffer**
Formamide (deionized, amberlite)	1 ml	0.1% bromophenolblue 25% Ficoll
Formaldehyde 35%	0.38 ml	1 mM EDTA
10 x Gel buffer		0.04 ml

2.7.4 Radioactive labelling of probes

The methods used for labelling are the same as those for Southern blotting. Probes used in Northern blots for *DEFH7* and *DEFH68* are identical to those used for Southern blots.

2.7.5 Hybridization

Pre-hybridization was carried out in a glass dish containing equal parts of solution A and B (5-10 ml each) and 100 µg/ml herring sperm DNA for 1-3 hr at 42°C, followed by addition of denatured probe for hybridization. The procedure was performed overnight under the same conditions; washing of membranes and detection of radioactive signals were performed in the same way as described above (paragraph 2.6.4) at 42°C.

Solution A	**Solution B**
10 x SSPE 10 x Denhardt's solution 1% SDS	Deionized formamide

2.8 RT-PCR

2.8.1 Reverse transcription (RT) using Superscript II

Total RNA from various *Antirrhinum* tissues was extracted using Total RNA Isolation Reagent (Biomol), and digested with DNase I (RNase-free; Roche) as follows:

Total RNA:	100 µl (50 µg)
10x DNase buffer:	20 µl
DNase (RNase-free):	1 µl (10 units)
H_20:	79 µl
Final volume	200 µl

The reaction was incubated at 37°C for 30 min to 1 hr and after that DNase I was removed by using the RNeasy plant minikit (Qiagen).

10 µg purified total RNA were heated for 5 min at 70°C, immediately chilled in ice and mixed to a reaction mixture containing (for each sample):

H_2O	14 µl
5 x first strand buffer (Gibco/BRL9)	10 µl
10 mM dATP, dGTP, dTTP, and 5 mM dCTP	2.5 µl each
[α^{32}P]-dCTP	1 µl
Oligo dT_{15}	1 µl (0.5 µg)
RNase inhibitor	0.5 µl
Superscript reverse transcriptase (Gibco/BRL)	1 µl (200 U)
Final volume:	30 µl

The resulting mixtures were treated as follows: 10 min at 20°C, 15 min at 37°C, 45 min at 42°C. 2 µl of 0.5 M EDTA were then added to each sample to stop the reaction and the synthesized first strand cDNA was precipitated with 50 µl 4 M NH_4OAc and 200 µl 100% EtOH for 30 min at room temperature, centrifuged for 30 min at 14,000 rpm and 20°C, washed with 80 % EtOH; after measuring the counts, the pellet was briefly dried by evaporation for 5-10 min, and dissolved in 100 µl TE for PCR.

2.8.2 PCR reaction

First strand cDNA	x µl (5 ng)
10 x buffer	5 µl
2 mM dNTPs	5 µl
0.2% Tween 20 (Sigma, P-1379)	5 µl
[α^{32}P]-dCTP (3000 Ci/mM)	0.5 µl
10 µM actin-specific primers or gene-specific primers	2 µl each
H_2O	x µl
Taq Polymerase	0.5 µl
Final volume	50 µl

2.8.3 PCR programme

a) for actin-specific primers ; b) for gene-specific primers

1:	95°C	1 min	1:	95°C	1 min
2:	94°C	40 sec	2:	94°C	40 sec
3:	57°C	30 sec	3:	60°C	30 sec
4:	72°C	40 sec	4:	72°C	20 sec
5:	72°C	1 min	5:	72°C	1 min
6:	15°C		6:		15°C

16 cycles (a) ; 33 cycles (b)

5 µl of each PCR reaction were loaded on 5% polyacrylamide gel. After electrophoretic separation, the gel was transferred to whatman paper, dried for 45 min on the gel dryer at 80°C and exposed overnight.

2.9 *In situ* hybridization

DNA templates used for preparing the probes were either cDNAs subcloned into pBluescript or were directly amplified by PCR. In both cases T3 polymerase was used to synthesize sense RNA and T7 polymerase for antisense RNA. A full-length *DEF* cDNA was subcloned into pGEM-T vector to generate a suitable template. The methods for digoxigenin labelling of RNA probes, tissue preparation and *in situ* hybridization were as described by Bradley et al. (1993).

2.10 Construction of phylogenetic trees

Phylogenetic trees were constructed based on published MADS-box sequences. The MADS-

domain sequences and the "170 domain" sequences which constitute the MADS domain and the subsequent 110 amino acids were used for construction as described in reference Theissen et al., 1996.

2.11 RsaI sublibrary construction, screening and sequencing

2.11.1 Sublibrary construction

A genomic library was screened using a mixture of 15 full-length MADS-box cDNAs from *Antirrhinum* as a probe. Filters were hybridized at both high (68°C) and low (42°C) stringencies. Phages which showed weak signals at high stringency, and stronger signals at low stringency were picked; a total of 60 candidates were obtained with insert sizes in the range of 12 to 23 kb. Furthermore, EcoRI was used to release the genomic fragments from the phage DNA and the resulting 60 genomic DNA inserts were pooled and digested with the frequent cutter RsaI. After electroelution, the fragments between 250 bp and 2.0 kb were subcloned in λNM1149, yielding the RsaI sublibrary.

2.11.2 Sublibrary screening

A RsaI sublibrary prepared from *Antirrhinum* plant genomic library λEMBL3 was screened essentially according to Sambrook and Russell (2001). *E. coli* host strain POP13 was used for λ phage infection and preparation.

2.11.2.1 l phage plating and transfer

An aliquot of a glycerol stock of *E. coli* POP13 was used to inoculate 50 ml LB medium containing 0.2% maltose and 10mM $MgSO_4$, the culture was grown with vigorous shaking at 32°C overnight. Bacteria were collected by centrifugation at 5,000 rpm for 10 min, and the pellet was resuspended in 5 ml 10 mM $MgSO_4$.

For phage plating, 300 µl of *E. coli* suspension was mixed with 2 x 10^5 pfu was mixed with 600 µl of SM buffer. The mixture was incubated for 15 min in a waterbath at 37°C, added to 40ml top agarose kept at 42°C, and plated on 10 90 mm Ø petri dishes containing NZ medium. The plates prepared for this screening were incubated at 37°C and stored at 4°C before membrane transfer.

Plaques were transferred onto nylon membranes, immediately followed by soaking membranes (phage side up) in denaturing solution for 10 min and neutralizing solution for 10 min; the membranes were dried at room temperature and the phage DNA was fixed to the membranes by baking at 80°C. The mixture of 15 full-length MADS-box cDNAs was

labelled with [α^{32}P]-dCTP and used as a probe to hybridize. The membranes were washed at low stringency (45°C). The procedure for hybridization and washing were performed as described above (2.6.4).

The 15 MADS-box cDNAs used were the following:

CDEF, cDEFH9, cDEFH11, cDEFH24, cDEFH49, cDEFH52, cDEFH57, cDEFH72, cDEFH76, cDEFH76B, cDEFH84, cFARINELLI, cGLOBOSA, cPLENA, cSQUA

SM solution

NaCl	
MgSO4. 7H2O	
1M Tris-Cl (pH 7.5)	50
2% (w/v) gelatin solution	5 ml
H_2O	to 1 liter

Top agarose

Agarose	0.6%
$MgSO_4$	10 mM

NZCYM medium

NZ amine	10 g
NaCl	5 g
Yeast extract	5 g
Casamino acids	1 g
$MgSO_4$. 7H2O	2 g
H_2O	to 1liter
pH to 7.5	
Bacto-Agar	15 g/l

Prehybridization/hybridization solution

SSPE	5 x
SDS	0.1%
Ficoll 400	0.02%
PVP	0.02%

2.11.2.2 Selection and rescreening of recombinant l phages

Positive candidates hybridizing with the probe were selected, taken out by using a pasteur pipette, released into an Eppendorf tube containing 1 ml SM solution and 3 drops of chloroform and mixed well; the λ phages were left to elute overnight at 4°C.

In order to get single plaques, serial dilutions (total 1:100000) of the phage suspension were performed in a total volume of 100 µl SM solution. The procedures for λ phage plating and transfer to the nylon membranes were the same as above (2.10.2).

The same mixture of full-length cDNAs as described above (2.10.2) was used as a probe to rescreen those positive plaques. The rescreening was performed until a single positive plaque was separated well from neighbouring plaques. Later on another round of rescreening was conducted to confirm that the single plaque picked up was the correct one.

2.11.2.3 PCR amplification of λ phage DNA

Inserts of the phage DNAs were amplified by PCR using an aliquot of each plaque suspension as template and insert flanking primer1: 5'-TGAGCAAGTTCAGCCTGGTTAAGTC-3' and primer2: 5'–GCTTATGAGTATTTCTTCCAGGGTA–3'. Amplified DNAs were loaded on 0.8% agarose gel to check the size and purified with Qiagen PCR purification kit. The PCR products were grouped by size; for fragments smaller than 500 bp in length manual sequencing analysis was performed, while bigger fragments were sent to the ADIS sequencing unit

PCR reaction

Plaque suspension	1 µl (about 5 ng DNA)
10 x buffer	5 µl
2 mM dNTPs	5 µl
10 µM primer 1	1 µl
10 µM primer 2	1 µl
DMSO	2.5 µl
H_2O	34 µl
Taq DNA polymerase (5 U/µl)	0.5 µl
Final volume:	50 µl

PCR programme

1:	95°C	1 min	
2:	95°C	30 sec	30 cycles
3:	60°C	30 sec	
4:	72°C	1.5 min	
5:	72°C	5 min	
6:	15°C		

2.11.3 DNA sequence analysis

DNA sequence determination was carried out using either the fmol DNA sequencing system (Promega) described above or an automated DNA sequencer (Model 377, Applied Biosystems). DNA sequence analysis was conducted using the MacVector (Oxford Molecular Group) and blast search programmes for sequence homology comparison.

2.12 Library screening

2.12.1 cDNA library screening

With the genomic RsaI fragments a screenings for full-length cDNAs of an *Antirrhinum* total plant cDNA library was carried out. A total of 6x 10^6 plaques were plated on 20 90mm Ø petri dishes in NZ medium. The procedures for plating, transfer, and rescreening were the same as for the RsaI sublibrary screening, but in this case partial putative MADS-box clones

were used as probes and hybridization and washing were performed at high stringency (68°C).

2.12.2 Genomic library screening

In order to elucidate the strutures of the newly identified MADS-box genes, screening of an *Antirrhinum* genomic library in λEMBL3 was conducted. *E. coli* strain K803 was used for phage infection and propagation, a total of 6x 10^6 plaques were plated on 20 90mm Ø petri dishes in NZ medium; the whole procedure for screening was the same as for cDNA library screening, but as probe the non-conserved region of the newly identified MADS-box cDNAs were used.

2.12.2.1 Extraction of l phage DNA

20 ml SM lysate buffer was added to a 12.5 cm x 12.5 cm square plate containing the confluent lysate obtained by infection of *E. coli* K803 with phages on NZ medium. The plate was gently shaken for 1-2 hr, 500 µl of chloroform were added to the collected lysate, and cell debris was precipitated by centrifugation at 5,000 rpm for 5 min. 25 µg of DNase I and 12.5 µg of RNase were added to the supernatant and incubated at 37°C for 30 min.

Phage particles were precipitated by incubation with 30% PEG 6000 in 1.5 M NaCl for 20 min on ice. After centrifugation at 10,000 rpm at 4°C for 20 min, the pellet was resuspended in 5 ml TE buffer supplemented with 200 µl 0.5 M EDTA, 100 µl 5 M NaCl and 250 µl 10% Triton X 100 and incubated at 65°C for 15 min. The phage DNA was extracted with 1 volume phenol/chloroform, 1 volume chloroform, and precipitated with 1/10 NaOAc and 1 volume isopropanol. The resulting pellet was washed with 70% EtOH, dried briefly, and dissolved in TE buffer.

In order to get more pure phage DNA, Qiagen columns for plasmid DNA isolation were used to purify the phage DNA again. The procedure was following the instruction supplied by the manufacturer.

2.13 PCR-based pools screening

2.13.1 Screening strategy

The reverse genetic screen was carried out as established at the John Innes Centre, Norwich, UK (E. Keck, R. Carpenter and E. Coen, unpublished). Integration of the transposable elements (Tam1,Tam2, Tam3, Tam4, Tam5, Tam6, Tam7, Tam8 and Tam9) into the genes *DEFH7* or *DEFH*68 was detected by hybridization of the *DEFH7* or *DEFH*68 probe to blots

of PCR products amplified with gene-specific primers and Tam element-specific primers, and confirmed by hybridization with the PCR product amplified with nested primers of the same Tam elements and the genes. Oligonucleotide primers used were: *DEFH7*-G-P1, 5'-ACT ATA CAG CTC TAG TTT TTG TCC-3'; *DEFH7*-G-P2, 5'-TAT TGC TGC CAG TAC GAG GTT TCA-3'; *DEFH7*-G-P1n, 5'-TCA GGA AAG GCA TAT CAG TAT GCA AG-3'; *DEFH7*-G-P2n, 5'-ATG GTT GTC CTT GAA ACA GGT CCA-3'; and Tam2,4,5,6, 5'-TC TTG GGA CAT AGG TTT TAT GCG ACA GAT-3' for *DEFH7*; *DEFH*68-N-P1, 5'-CAC TAT AGA GCG ATA CCA ATG TCA CA-3'; *DEFH*68-N-P2, 5'-AAG TTC CAT ACA ACT GCA ACA AGC A-3'; *DEFH*68-N-P1n, 5'-CCA GCA TTG AAC TTA CAG AAA GCG-3'; *DEFH*68-N-P2n, 5'-ATT TCA TGC TAG TTT CCA AGC G-3' for *DEFH68*. The pools were screened as 66 superpools containing each DNAs from 450 plants, each of which was created by combining 3 subpools of genomic DNA prepared from the leaves of 150 plants. Positives were first confirmed on the subpools of 150 plants and subsequently on 10 new subpools generated from batches of 15 plants separately, which contributed to the positive subpool. Finally, seeds collected from the 15 plants in each positive pool were sown and genomic DNA was isolated pooling it from the seedlings of each row or column in a tray. PCR products amplified from those genomic DNAs with the same combination of primers were tested by Southern blot analysis to confirm Tam element integration into *DEFH7* and *DEFH*68, respectively. Furthermore, PCR amplification using single plant genomic DNA from the positive row or column plant pool was tested again to find out single plants for Tam element insertion.

PCR was carried out in 25 µl reaction and ran as follows: 1 min at 95°C followed by 35 cycles of 40 sec at 94°C, 30 sec at 55°C, and 1.5 min at 72°C, then 2 min at 72°C. 3 µl of each superpool reaction were dot-blotted onto a nylon membrane and the filter was briefly dried at room temperature, baked at 80°C for 30 min and hybridized at 68°C with a ^{32}P-labelled c*DEFH7* or c*DEFH*68 probe. Subsequent subpool screening was done by Southern blotting of the PCR products.

2.13.2 Genomic DNA isolation from transposon mutagenized *Antirrhinum* plants

A small piece of a plant leaf was collected in a sterile Eppendorf tube containing 400 µl of elution buffer and ground with a macerator. 1 volume of phenol/chloroform was added to each sample, mixed well and left at room temperature for more than one hour. The extracted DNA was separated by centrifugation at 14,000 rpm for 5 min, 200 µl of supernatant were precipitated with 1 volume of isopropanol and finally the pellet was washed with 70%

ethanol and resuspended in 100 µl of TE buffer.

Elution buffer	
Tris-cl pH7.5	200 mM
NaCl	250 mM
EDTA	25 mM
SDS	0.5%

2.14 Yeast two-hybrid screening

2.14.1 Plasmid construction for two-hybrid screening

All baits were constructed by inserting PCR fragments into the plasmid vector pGBT9. The PCR fragments were derived from the relevant cDNAs and generated using primers into which appropriate restriction enzyme sites were incorporated. In all cases the construction of the bait resulted in the in-frame fusion, as confirmed by sequencing analysis of the entire coding regions of the various genes and of the GAL4 DNA binding domain. The oligonucleotide primers used were as follows: *DEFH7* 5'-GCG AAT TCA TGG GAA GAG GTA AAG-3', 5'-CAG GAT CCT TAA TGG TTG TCC TTG-3'; *DEFH*68 5'-GCG AAT TCA TGG TGA GAG GAA AGA-3', 5'-AAG GAT CCT CAT TGC TGG AGT GGA-3'. The coding sequences were also cloned into the plasmid pGAD424 containing the Gal4 activation domain to test for homodimerization and possible self-activation. The plasmid construction was performed as previously described (Davies et al., 1996). The inserts in all clones were fully sequenced.

2.14.2 Optimal concentration of 3-amino-1, 2,4-triazole (3-AT) in two-hybrid screens

The yeast host strainY190 is leaky for histidine expression in the absence of 3-AT. In order to identify the reasonable concentration of 3-AT to suppress background growth of the strain Y190, the Y190 (BD/DEFH7 and BD/YS68) colonies were tested on a series of plates containing YPD medium supplemented with different concentrations of 3-AT (0, 5, 10, 15, 20, 25, 30, 35, 40, 45, and 50 mM). 25 mM 3-AT in the medium turned out to be sufficient to reduce background growth of the strain Y190.

2.14.3 Two-hybrid screen

The predominant *DEFH7* (*DEFH*68) cDNA was amplified using oligonucleotide primers to introduce cloning sites. Amplification was carried out using the same oligonucleotide primers mentioned above (2.14.1). PCR fragments were digested with *Eco*RI and *Bam*HI and ligated

into the vector pGBT9 digested with the same restriction enzymes to form the bait. The bait included the whole coding sequences. Two-hybrid screens were carried out according to the method described by Davies *et al*. (1996).

Interactions were investigated both by screening an *Antirrhinum* total plant cDNA yeast expression library and by directly testing for interaction between the bait and previously isolated MADS-box preys.

2.14.3.1 b-galactosidase filter assay

Nylon filters were divided into sectors with each sector numbered corresponding to the colonies to be tested. Colonies were streaked onto the filter placed onto a Y-SD plate with appropriate selection and allowed to grow for one or two days at 30°C. The filters were put into liquid nitrogen for 5 sec, placed onto a paper filter soaked with 1.8ml assay buffer in the lid of a petri dish, and the petri dish was sealed with parafilm and incubated at 37°C. Blue colour representing interactions developed after 20 min to 30 hr.

2.14.3.2 Grouping positive colonies from the b-galactosidase test

Yeast plasmids were isolated from putative positive colonies. Flanking primers of the pGAD424 vector were used to amplify inserts. PCR products were Southern blotted to nylon membrane and hybridized at 68°C with the biggest insert from those positive colonies selected based on β-galactosidase analysis. After strip washing, the same filter was reused for hybridization at 68°C with a labelled cDNA from another colony, which previously did not hybridize. By this approach colonies were assigned to different groups. Then, one member of each group with the biggest insert was used for sequence analysis.

b-galactosidase assay buffer		**Z-buffer**	
Z-buffer	10 ml	$Na_2HPO_4.\ 2H_2O$	11.1 g
β-mercaptoethanol	27 µl	$NaH_2PO_4.\ H_2O$	5.5 g
X-gal (20 mg/ml in DMF)	167 µl	KCl	0.75 g
		$MgSO_4.\ 7H_2O$	0.25 g
		Add H_2O to 1 liter	
		Adjust to pH7	

2.14.3.3 Small scale transformation in yeast

A single fresh colony was scraped from a plate into 10ml of appropriate selective medium and grown overnight at 30°C. The culture was diluted in the same medium according to the number of transformations (using 5ml per transformation) and regrown for 2 hr. The final

culture was pelleted and washed with water, 1ml of 1 x LiAc/TE, and resuspended in 1 x LiAc/TE of 100 µl per transformation. The suspension was added to a mixture of 1 µg plasmid DNA, 10 µg salmon sperm carrier DNA, 300 µl PEG/LiAc/TE, and 5 µl DMSO. The mixture was first incubated at 30°C for 30 min, then at 42°C for 15 min and a 100 µl aliquot was plated on selective plates.

1x LiAc/TE		**10x TE**	
10 x LiAc	100 µl	1 M Tris-HCl pH7.5	100 ml
10 x TE	100 µl	500 mM EDTA pH7.5	20 ml
Sterile water	800 µl	add H_2O to 1 liter	
1x PEG/LiAc/TE		**10x Lithium acetate**	
10 x LiAc	500 µl	Lithium acetate	40.8 g
10 x TE	500 µl	add H_2O to 400 ml	
50% (W/v) PEG 4000	4 ml	Adjust to pH 7.5	

2.14.3.4 Plasmid DNA isolation from yeast

Lysis solution:		**10X Dropout**	
Triton X-100	2%	L-Isoleucine	300 mg
SDS	1%	L-Valine	1500 mg
NaCl	100 mM	L-Adenine hemisulphate salt	200 mg
Tris pH 8.0	10 mM	L-Arginine HCl	200 mg
EDTA	1.0 mM	L-Histidine HCl monohydrat	200 mg
		L-Leucine	1000 mg
YPD medium:		L-Lysine	300 mg
Peptone/Tryptone	20 g	L-Methionine	200 mg
Yeast Extract	10 g	L-Phenylalanine	500 mg
Adjust to pH 5.8		L-Threonine	2000 mg
Add H_20 to 950 ml and autoclave		L-Tryptophan	200 mg
Before use add 50 ml of 40% dextrose		L-Tyrosine	300 mg
		L-Uracil	200 mg
Y-SD selection medium:		Add H_20 to 1 liter	
Yeast Nitrogen Base w/o aa.	6.7 g		
Agar	20 g		
Adjust to pH 5.8			
Add H_20 to 850 ml and autoclave			
Before use add:			
40% dextrose	50 ml		
10 x Dropout	100 ml		

A single colony picked from Y-SD plates with L-Tryptophan and L-Leucine dropout medium (–TL plates) was inoculated in 5 ml Y-SD medium with L-leucine dropout (-L medium) overnight at 30°C. 100 µl of culture were used to inoculate 5 ml fresh –L medium, grown

overnight and again 100 µl of the second culture was used to inoculate 5 ml YPD medium and grown overnight. 1 ml of the final culture was pelleted and lysed in lysis solution. The lysate was mixed with 1 volume phenol/chloroform and 100 mg washed glass beads in a DNA mixer for 5-10 min. The mixture was centrifuged and the supernatant was precipitated with 1/10 volume of NaOAc and 1 volume of isopropanol. The pellet was briefly washed with 70% ethanol and dissolved in TE buffer.

2.15 Constructions for plant transformation

2.15.1 Constructions for overexpression in plant transformation

Gene fusions of the CaMV35S promoter to the *DEFH7* (*DEFH*68) coding region in sense and antisense orientation were constructed in the *Agrobacterium* binary vector pH35SXS for *Antirrhinum* transformation and vector pB35SXS for transformation of *Arabidopsis*. Appropriate PCR fragments carrying its endogenous start and stop codons were generated at the 5' or 3' end by amplification with primers containing, respectively, artificially introduced terminal XbaI and XhoI sites; Primers for sense orientation: *DEFH*7-XbaI-S1, 5'-GCT CTA GAA TGG GAA GAG GTA AAG TAG AG-3', *DEFH*7-XhoI-S2, 5'-CCC TCG AGT TAA TGG TTG TCC TTG AAA CAG-3'; *DEFH*68-XbaI-S1, 5'-GCT CTA GAA TGG TGA GAG GAA AGA CTC AGA-3', *DEFH*68-XhoI-S2, 5'-CCC TCG AGT CAT TGC TGG AGT GGA CGC TTA-3'; Primers for antisense orientation: *DEFH*7-XbaI-A1, 5'-GCT CTA GAA TGG TTG TCC TTG AAA CAG G-3', *DEFH*7-XhoI-A2, 5'-CCC TCG AGA TGG GAA GAG GTA AAG TAG A-3'; *DEFH*68-XbaI-A1, 5'-GCT CTA GAT TGC TGG AGT GGA CGC TTA G-3', *DEFH*68-XhoI-A2, 5'-CCC TCG AGA TGG TGA GAG GAA AGA CTC A-3'. The PCR product was digested with XbaI and XhoI and cloned in both orientations (sense/antisense) into the unique polylinker of the CaMV35S promoter/terminator casette of pH35SXS and pB35SXS, respectively. The resulting plasmids were called pB-35S::DEFH7se, pB-35S::DEFH68se (sense for *Arabidopsis*), pH-35S::DEFH7se, pH-35S::DEFH68se (sense for *Antirrhinum*); pH-35S::DEFH7as, pH-35S::DEFH68as (antisense for *Antirrhinum*). These plasmid DNA inserts were fully sequenced. Transformation of binary vectors into the *A.tumefaciens* strain GV3101 harbouring plasmid pMP90 was performed by electroporation.

2.15.2 Constructions for complementation of the *soc1* mutant and overexpression in wild-type *Arabidopsis* plants

The full-length c*DEFH*68/c*DEFH*24 were amplified by PCR with primers containing

artificially introduced terminal BamHI sites. The primers were: 5'-CAG GAT CCA TGG TGA GAG GAA AGA- 3' and 5'-CAG GAT CCT CAT TTT TGG GGT GGA-3' (used for the amplification of *cDEFH*24) and 5'-CAG GAT CCA TGG TGA GAG GAA AGA-3' and 5'-AAG GAT CCT CAT TGC TGG AGT GGA-3' (used for the amplification of *cDEFH*68). The PCR resulting products were digested with BamHI, gel purified, cloned into BamHI-linearized binary vector pPCV702 to produce sense cDNA construts; the constructs were transformed into Agrobacterium strain GV3101 and used later for both *soc1* mutant and wild type *Arabidopsis* transformations.

2.16 Plant transformation and regeneration

2.16.1 Plant transformation and regeneration in *Antirrhinum*

The *A. tumefaciens* containing chimeric constructs with the gene of interest were grown overnight with shaking at 28°C in 100 ml YEB medium. The pellet of 40 ml of the overnight culture was resuspended in 2 ml of BM supplemented with 25 mg/ml indolebutyric acid. The remaining hypocotyls of the seedlings which had grown for 4-6 weeks in MS medium were infected with the Agrobacteria suspension and incubated in the dark at 25°C for 3-5 days. The selection procedure was as follows: hypocotyls excised from the roots were transferred onto selective H1 medium; after 4-6 weeks surviving calli were transferred to H2 medium to improve their growth. The calli with a diameter of 1-2 cm were transferred to H3 medium, 3-4 weeks later to H4 and 4 weeks later to H5 medium. After transfer to both H4 and H5 media, calli were incubated for 2 days in the dark. The first shoots were visible after 3-4 weeks on H5 medium. Regenerated shoots were excised, rooted on hormone-free MS medium, and transferred to the greenhouse. The detailed description and media composition were described in Heidmann *et al*. (1998).

2.16.2 Plant transformation in *Arabidopsis*

A single colony of *Agrobacterium* grown in YEB plates containing antibiotics was incubated with shaking in 5 ml YEB medium overnight at 28°C; the next day, the culture was diluted to 50 ml and incubated overnight, and furthermore diluted to 500 ml and incubated overnight. The cells were harvested from the final culture and resuspended in infiltration medium supplemented with 50 µl/L of 0.005% v/v Silwet. The plants for transformation were placed in short-day condition for 2 weeks after germination in order to get larger rosettes, then grown in long-day condition. The plants were dipped in the infiltration medium about 1 week

after the primary inflorescences were clipped and the plants were covered with a lid or a plastic bag for 1-2 days. The seeds were collected two to three weeks after transformation.

Infiltration medium	
1/2 x Murashige & Skoog salts	4.4 g/L
1 x B5 vitamins	
Sucrose	5.0%
Benzylamino Purine	0.044 µM
Adjust pH with KOH to 5.7	

2.16.3 Generation of transgenic lines of overexpression in *Arabidopsis*

A.thaliana transformants as well as their progenies were selected with 0.1% Basta once a week in the first two weeks after germination. Transgenic plants were grown in a greenhouse at 18-25°C with additional light during winter in long day condition (LD, 16 hr light/8 hr dark). Plants were grown in 10 cm plastic trays filled with ready-to-use commercial, prefertilized soil mixture (Type ED73, Werkverband EV).

2.16.4 Generation of transgenic lines of the *soc1* mutant

Microcentrifuge tubes containing 100-150 seeds per line were placed inside a dessicator jar, containing 100 ml bleach in a beaker. Immediately after addition of 3 ml of concentrated HCl, the jar was sealed to fumigate seeds overnight in a sterile laminar flow hood. The sterilized seeds were plated on MS/0.8% tissue culture Agar plates with 50 µg/ml kanamycin; the plates were kept in the dark at 4°C for 2 days and then moved to continuous light (50-100 µE $m^{-2}sec^{-1}$) for 7-10 days, and the putative transgenic plants were transferred to soil and grown for further analyses.

RESULTS *CHAPTER 3*

3.1 Isolation and structural characterization of new MADS-box genes in *Antirrhinum*

DEFICIENS, one of the four founding members of the MADS-box gene family, was isolated from *Antirrhinum* by differential screening of a cDNA library with a single strand probe obtained by subtracting wild type cDNA with *def* mutant mRNA (Sommer et al., 1990). Subsequently, about 24 members of the family have been identified in *Antirrhinum* by screening of cDNA libraries at low stringency with probes containing a mixture of previously isolated family members as a probe or by detecting them as partners interacting with known MADS-box proteins in yeast two-hybrid screens (Huijser et al., 1992; Tröbner etal., 1992; Zachgo et al., 1997; Davies et al., 1996). The number of MADS-box genes in the genome, however, appears to be much larger, as indicated by the over 80 entries for *Arabidopsis* in the database (http://www.ebi.acuk/interpro/). The main goal of this thesis therefore was to get hold of a large collection of *Antirrhinum* MADS-box family and to study the function of some of them during development.

3.1.1 Screening of a genomic library

Screening for new MADS-box genes in cDNA libraries has some disadvantages. One of these is that cDNA libraries are biased by the highly variable abundance of different mRNAs leading to failure of detection of transcripts expressed at a very low level; another is that very likely not all tissues and developmental stages are represented in the mRNA population used to construct the cDNA library. Screening of a genomic library is more promising because all genes are present at an equal ratio. Therefore, a genomic library in phage λEMBL4 was probed at low stringency with a mixture of cDNA fragments of 15 different MADS-box family members (see 2.11.2.1). Two rounds of hybridizations were carried out with the same set of nitrocellulose filters. In the first round of screening hybridisation was undertaken at low stringency (50°C) to obtain related MADS genes; the second round of screening used more stringent conditions (68°C) to exclude already known genes. Sixty recombinant lambda phages assumed to contain new MADS-box gene sequences were obtained containing approximately 12 kb to 23 kb long genomic DNA inserts.

The 180 bp long region containing the MADS box is difficult to identify within a 12 to 23 kb long phage insert. To circumvent this problem the phage inserts were isolated, pooled and digested with the frequently cutting restriction enzyme RsaI to obtain relatively short

fragments. The resulting fragment mixture was cloned into the insertion vector λNM1149, and this 'RsaI sub-library' was screened again at low stringency using the mixture of 15 known MADS-box sequences as a probe. Fifty five recombinant phages assumed to carry the MADS-box within their RsaI insert were identified.

The size of the MADS-box-containing phage inserts served as the criterium to distinguish between MADS sequences derived from different genes and to group identical clones. Twelve groups with one to 17 members were identified this way, and one member of each group was sequenced. The twelve sequences were compared to the DNA sequences of the known *Antirrhinum* MADS-box genes to determine whether they represented new genes. Five of the groups contained already known MADS-box genes, four groups contained sequences that were not related to the MADS-box, and the remaining three groups represented novel MADS-box genes, named *DEFH7*, *DEFH17* and *DEFH20*, according to the isolation number of the sequenced phages.

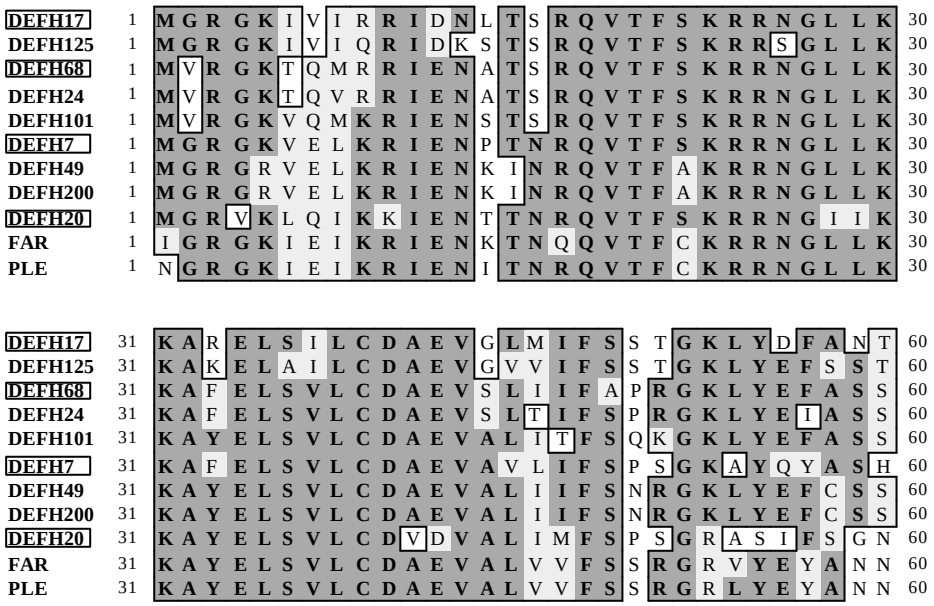

Figure 3-1. Amino acid sequence alignment of the 60 N-terminal amino acids encoding the MADS-box from *Antirrhinum* MADS-box factors. The names of the newly isolated MADS-box family members are in boxes. Dark and light shading indicates identical and conserved amino acids, respectively.

The entire MADS-box is encoded by one exon and so the genomic MADS-box sequence can easily be translated into the protein sequence. The open reading frames of the new MADS-box genes are shown in Fig.3-1, together with the closest related known *Antirrhinum* MADS-

box amino acid sequences. This compilation reveals the high degree of sequence similarity at the amino acid level, characteristic for the MADS-box in general, and deviations between the compiled sequences, characteristic for individual proteins.

3.1.2 Isolation of full-size cDNAs of the new MADS genes

The genomic sequences represented only a short segment of the *DEFH7* and *DEFH68* genes due to the strategy applied to isolate the new MADS-box genes. To get more sequence information on the corresponding transcripts, a cDNA library derived from total plant mRNA has been screened with the *Rsa*I fragments as probes, along with a probe containing *DEFH68*, previously identified as a partial cDNA in a screen for ternary factors interacting with the DEF/GLO heterodimer (Egea-Cortines, unpublished data).

Six plaques hybridising to the *DEFH7* probe and twelve hybridising to *DEFH68* were obtained from 3x 10^5 plaques plated. The longest cDNA insert identified for *DEFH7* was 795 bp long with an open reading frame of 206 amino acids, while the putative *DEFH68* protein was 284 amino acids long as derived from the longest DEFH68 cDNA which contained 1022 bp (Figures 3-2A and 2B). No positive clones could be identified for *DEFH17* and *DEFH20*, indicating either that the respective genomic sequences encode untranscribed pseudogenes or that transcripts of these genes were not present in the library. *DEFH17* and *DEFH20* were not further characterized in this thesis.

A

```
ATGGGAAGAGGTAAAGTAGAGTTGAAGAGAATTGAGAATCCGACAAACAGACAAGTGACGTTTTCAAAGAGAAGAAATGGCTTGCTAAAG 90
 M  G  R  G  K  V  E  L  K  R  I  E  N  P  T  N  R  Q  V  T  F  S  K  R  R  N  G  L  L  K

AAAGCTTTTGAACTGTCTGTACTTTGTGATGCTGAGGTTGCTGTTCTTATCTTCTCTCCTTCAGGAAAGGCATATCAGTATGCAAGTCAT 180
 K  A  F  E  L  S  V  L  C  D  A  E  V  A  V  L  I  F  S  P  S  G  K  A  Y  Q  Y  A  S  H

GACACGCATAGGACAATTGCAAGGTATAAAAGTGAAGTTGGAATAACCAAACCAGGTGACCAGGGCATCACATCCATGGAGGTTTGGAGA 270
 D  T  H  R  T  I  A  R  Y  K  S  E  V  G  I  T  K  P  G  D  Q  G  I  T  S  M  E  V  W  R

AATGAAATTGAAGACTTAAAAAGAACTGTTGATGCCCTGGAAGCAAGAGATATGCATTTTGCTGGAGAAAACTTATCAGGATTAGGCATG 360
 N  E  I  E  D  L  K  R  T  V  D  A  L  E  A  R  D  M  H  F  A  G  E  N  L  S  G  L  G  M

AAAGACCTTAAACAGTTAGAACGGCAGATAAGAATTGGGGTGGAACGTATTCGCTCTAAAAAGAGGCGTATCATCGCAGAACACATGACT 450
 K  D  L  K  Q  L  E  R  Q  I  R  I  G  V  E  R  I  R  S  K  K  R  R  I  I  A  E  H  M  T

TATCTGAAGAAACGGCATAAAGACCTACAAGAAGAGAACAACAATCTCCAAAAGAGAGTCAAGCTACATGAAGTTCAAGAGGCCAACACA 540
 Y  L  K  K  R  H  K  D  L  Q  E  E  N  N  N  L  Q  K  R  V  K  L  H  E  V  Q  E  A  N  T

AGCTGCTCAATCATTTATGACTCAGATGGAACCAGGGTATTCCCAGGGTTTTCTTGGACCTGTTTCAAGGACAACCATTAA 621
 S  C  S  I  I  Y  D  S  D  G  T  R  V  F  P  G  F  S  W  T  C  F  K  D  N  H  *
```

B

```
ATGGTGAGAGGAAAGACTCAGATGAGGCGTATAGAAAACGCGACAAGCAGACAAGTGACCTTCTCTAAAAGGAGGAATGGTCTTCTTAAA 90
 M  V  R  G  K  T  Q  M  R  R  I  E  N  A  T  S  R  Q  V  T  F  S  K  R  R  N  G  L  L  K

AAAGCTTTTGAGCTTTCAGTTCTTTGTGATGCTGAGGTTTCTCTCATTATATTTGCACCCAGAGGCAAGCTCTATGAATTTGCAAGTTCA 180
 K  A  F  E  L  S  V  L  C  D  A  E  V  S  L  I  I  F  A  P  R  G  K  L  Y  E  F  A  S  S
```

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AGCATGCAGGACACTATAGAGCGATACCAATGTCACACTAAAGAACTTCAAGCGAATAATCCGCCTGCTGAACATAATATACAGCACGTA 270
 S  M  Q  D  T  I  E  R  Y  Q  C  H  T  K  E  L  Q  A  N  N  P  P  A  E  H  N  I  Q  H  V

AGGCACGAAGCAGCTAGTTTGATGAAAAAGATAGAGCAACTTGAGACTTCAAAACGGAAGTTACTTGGGGAAGGTCTGGGAACATGCACC 360
 R  H  E  A  A  S  L  M  K  K  I  E  Q  L  E  T  S  K  R  K  L  L  G  E  G  L  G  T  C  T

TTTGAAGAACTGCAGCAGTTAGAACAACAGTTGGAACGCAGTGTCGCTACCATTCGTGCAAGAAAGACGCAAATGTTCAAGCAGCAGATT 450
 F  E  E  L  Q  Q  L  E  Q  Q  L  E  R  S  V  A  T  I  R  A  R  K  T  Q  M  F  K  Q  Q  I

GAACAATTGAAAGAAAAGGGAAAATCCCTAGCTGCTGAAAATGCCATGCTCCATCAGAAGATTGGAGTGGAACAGCAACAAGTACCAGCA 540
 E  Q  L  K  E  K  G  K  S  L  A  A  E  N  A  M  L  H  Q  K  I  G  V  E  Q  Q  Q  V  P  A

TTGAACTTACAGAAAGCGGTTATGGGTTCCTCGGAGATTAGTGAAGTTTCGGATGTGGAGACTGAATTGTTCATTGGACTGCGTGAAACC 630
 L  N  L  Q  K  A  V  M  G  S  S  E  I  S  E  V  S  D  V  E  T  E  L  F  I  G  L  R  E  T

AGGGCTAAGCGTCCACTCCAGCAA 654
 R  A  K  R  P  L  Q  Q
```

Figure 3-2. cDNA sequence and deduced amino acid sequence of DEFH7 (A) and DEFH68 (B). The MADS-box is in the shaded boxes and K-box is underlined.

3.1.3 Structure and copy number of the *DEFH7* and *DEFH68* genes

The copy number of *DEFH7* and *DEFH68* in the *Antirrhinum* genome was determined by Southern blot experiments. A single hybridising band could be detected under stringent hybridization conditions, irrespective of the enzymes used to digest the genomic DNA (Figure 3-3). It appears, therefore, that both *DEFH7* and *DEFH68* are single copy genes.

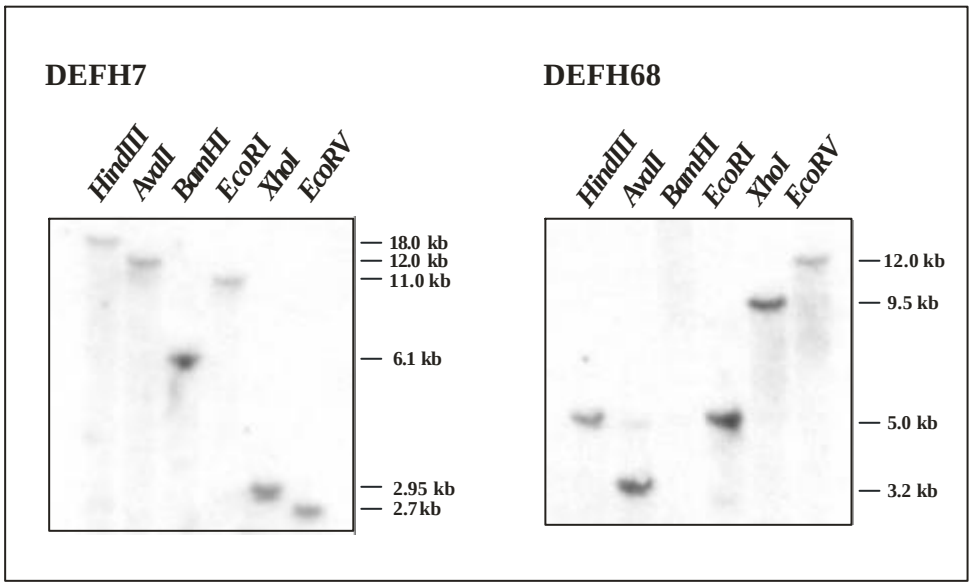


Figure 3-3. Copy number of the *DEFH7* and *DEFH68* genes. Southern blots shown in the figure were prepared from 4 ug of *A. majus* genomic DNA digested with the restriction enzymes indicated above the lanes. For hybridisation at high stringecy radioactively labelled gene-specific cDNA fragments were used as probes. The size of the fragments is indicated at the right.

To elucidate the structure of the *DEFH7* and *DEFH68* genes, a genomic library was screened with the C-terminal part of the respective cDNAs as a specific probe. Five positive clones

were identified for each gene. To obtain the shortest possible region that contains the entire coding region of the genes the phage inserts were amplified by PCR using primers derived from the 5' and the 3' ends of the longest corresponding cDNA sequences.The *DEFH7* gene could easily be amplified and subsequently sequenced this way. Unfortunately, however, the *DEFH68* gene could not be amplified.

This analysis revealed that the structural part of the *DEFH7* gene is 1624 bp long. In spite of its unusually small size in comparison to other MADS-box genes, the *DEFH7* gene consists of 8 exons separated by 7 introns (Figure 3-4), similar to the exon/intron structure characeristic for many MADS-box genes (Ma et al., 1991; Huijser et al., 1992). Furthermore, like in many other MADS-box genes, the fifth and sixth exon of *DEFH7* encode 42 amino acids each (Huijser et al., 1992). Interestingly, the first intron of *DEFH7* is much smaller than that of other MADS-box genes, for instance, that of SQUA (Fig. 3-4). It is not clear whether this size and structure is characteristic for genes related to DEFH7, because no data are available for its most closely related homologue, TM8, from tomato (see next chapter).

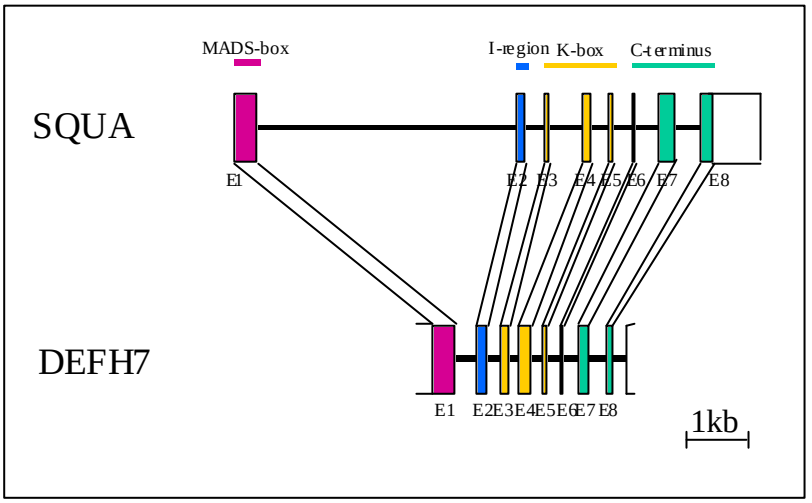


Figure 3-4. Comparison of the exon-intron structure of the *DEFH7* and *SQUA* genes. Exons are numbered and presented in boxes, connected by horizontal lines representing introns. The colors indicate different regions of the MADS-box genes shown above the structure and white boxes represent the untranslated regions. Open boxes in the *DEFH7* gene indicate that no information is available about the start and the end of transcription. Horizontal lines connect conserved positions between the two MADS-box genes.

3.2 Phylogenetic relationships

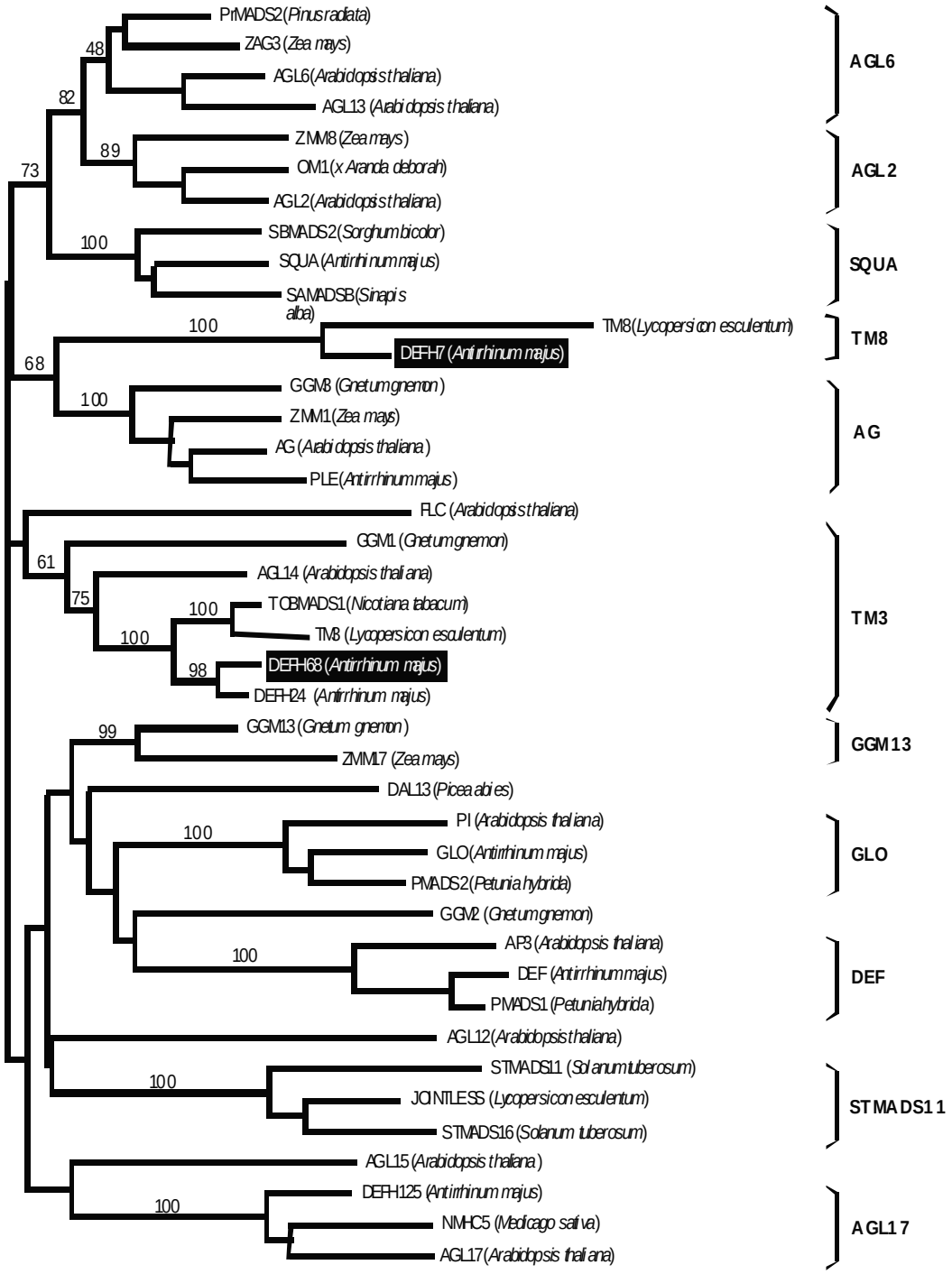
Phylogenetic reconstruction of MADS-box genes revealed that this multigene family consists of several distinct subfamilies or clades (Purugganan et al., 1995; Theissen et al., 1996). In many cases, subfamily members share similar expression patterns and related functions (Theissen et al., 1996). Therefore, phylogenetic analysis might give a first hint towards the function of the new MADS-box genes and indicate their evolutionary relation to other MADS-box genes.

To determine their relation to MADS-box factors annotated in the database (http://www.ncbi.nlm.nih.gov/), a phylogenetic tree was constructed with DEFH7 and DEFH68. The phylogenetic tree shown in Figure 3-5A was based on the first 170 amino acids of the proteins which comprises the M, I and K domains of the MADS proteins. The least conserved C-terminal region was excluded from the analyses (see 1.1.1.3), because the C domain is too divergent to be reliably aligned between members of different subfamilies (Theißen et al., 1996).

According to these analyses, DEFH7 constitutes a new subfamily with TM8, a MADS-box gene isolated from tomato, which is expressed in the inner three whorls of the tomato flower (Pnueli et al., 1991). The grouping of these two genes is supported by a bootstrap of 100%, which means a strong reliability for the correctness of calculation of this grouping. The amino acid sequences of the two proteins share 64% identity over the entire sequence (Figure 3-6A). Interestingly, no close homologue of DEFH7 appears to exist in *Arabidopsis*.

DEFH68 belongs to the TM3 subfamily, which contains several members derived from different species. In order to define the exact relationship among TM3 subfamily members and DEFH68, an additional tree was constructed with DEFH68 and the TM3-related MADS-box proteins from angiosperms and gymnosperms.. This analysis, also based on the first 170 amino acids of the proteins, revealed that DEFH68 is highly homologous to the *Antirrhinum* DEFH24 protein (Figure 3-5B). Their closest orthologue in *Arabidopsis* is SOC1, a flowering time gene (Lee et al., 2000). Amino acid sequence comparison showed 82% identity between DEFH68 and DEFH24, and 67% identity between DEFH68 and SOC1 (Figure 3-6B). Almost all TM3 family members are ubiquitously expressed in vegetative and reproductive tissues of the plant (Theißen et al., 1996), and so it can be speculated that *DEFH68* and *DEFH24* might also have similar expression patterns and functions.

A


PrMADS2 (Pinus radiata)
ZAG3 (Zea mays)
AGL6 (Arabidopsis thaliana)
AGL13 (Arabidopsis thaliana)
ZMM8 (Zea mays)
OM1 (x Aranda deborah)
AGL2 (Arabidopsis thaliana)
SBMADS2 (Sorghum bicolor)
SQUA (Antirrhinum majus)
SAMADS8 (Sinapis alba)
TM8 (Lycopersicon esculentum)
DEFH7 (Antirrhinum majus)
GGM8 (Gnetum gnemon)
ZMM1 (Zea mays)
AG (Arabidopsis thaliana)
PLE (Antirrhinum majus)
FLC (Arabidopsis thaliana)
GGM1 (Gnetum gnemon)
AGL14 (Arabidopsis thaliana)
TOBMADS1 (Nicotiana tabacum)
TM3 (Lycopersicon esculentum)
DEFH68 (Antirrhinum majus)
DEFH24 (Antirrhinum majus)
GGM13 (Gnetum gnemon)
ZMM17 (Zea mays)
DAL13 (Picea abies)
PI (Arabidopsis thaliana)
GLO (Antirrhinum majus)
PMADS2 (Petunia hybrida)
GGM2 (Gnetum gnemon)
AP3 (Arabidopsis thaliana)
DEF (Antirrhinum majus)
PMADS1 (Petunia hybrida)
AGL12 (Arabidopsis thaliana)
STMADS11 (Solanum tuberosum)
JOINTLESS (Lycopersicon esculentum)
STMADS16 (Solanum tuberosum)
AGL15 (Arabidopsis thaliana)
DEFH125 (Antirrhinum majus)
NMHC5 (Medicago sativa)
AGL17 (Arabidopsis thaliana)
48
82
89
73
100
100
68
100
61
75
100
100
98
99
100
100
100
100
AGL6
AGL2
SQUA
TM8
AG
TM3
GGM13
GLO
DEF
STMADS11
AGL17

B

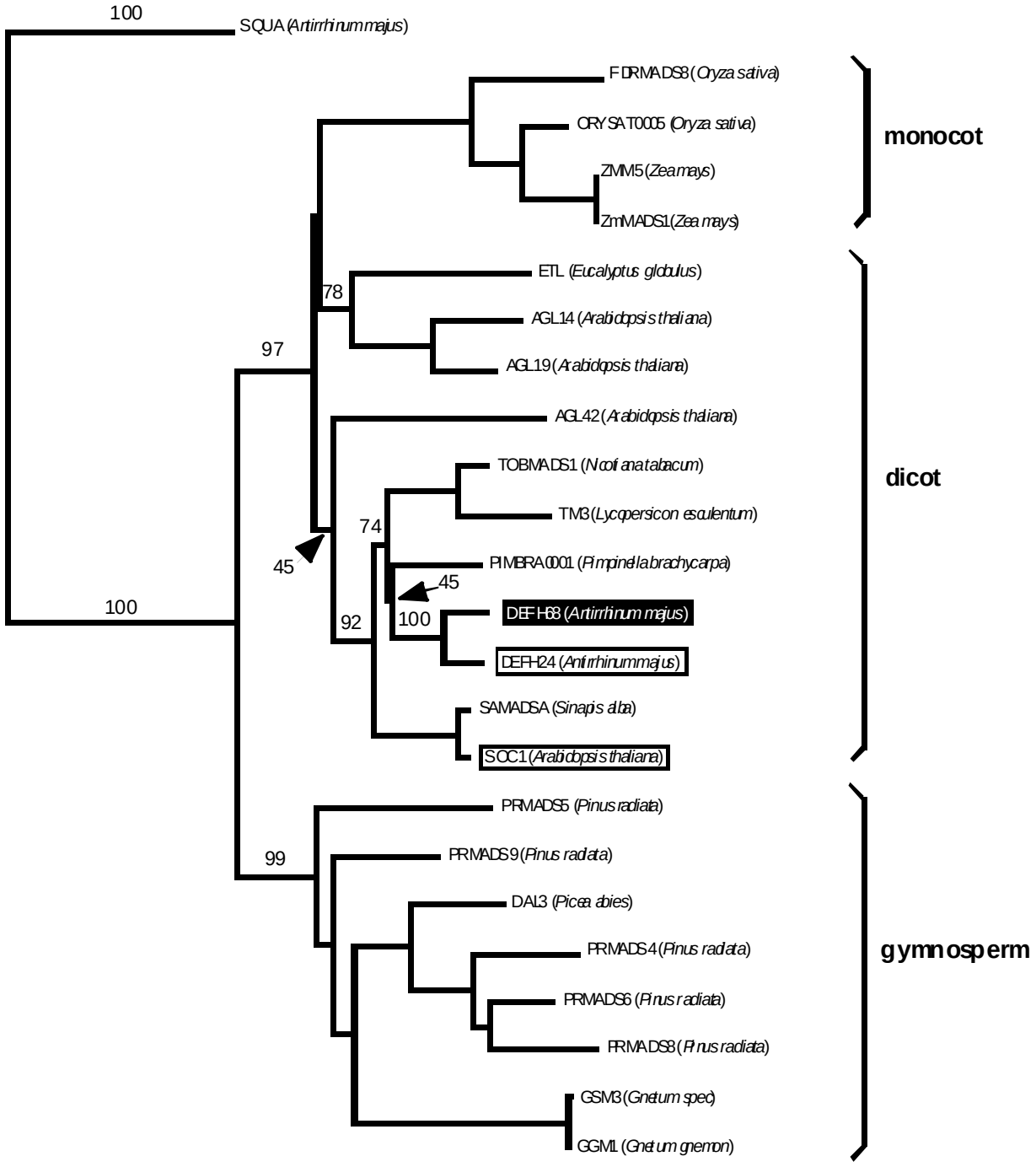
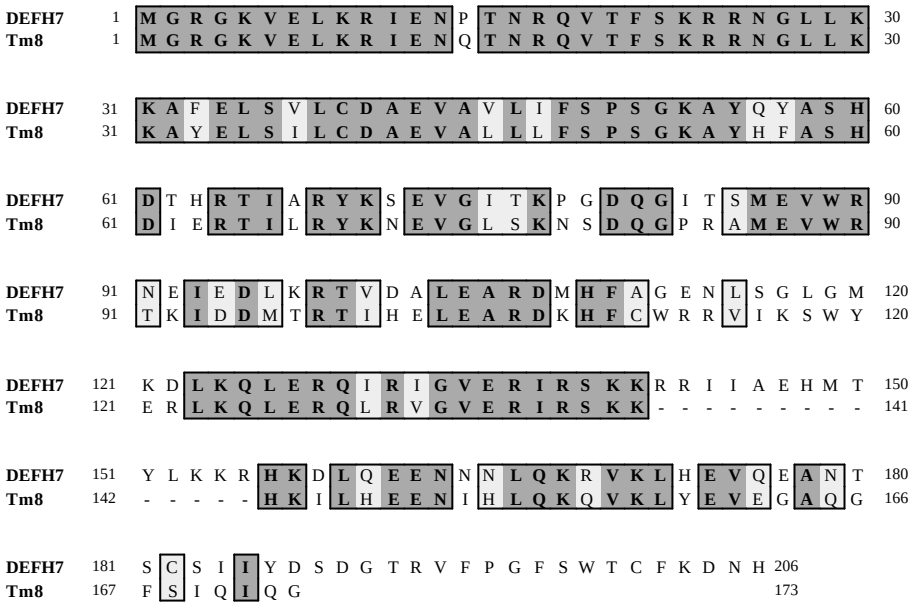


Figure 3-5. Phylogenetic trees with the newly isolated *Antirrhinum* MADS-box genes *DEFH7* and *DEFH68*. Genus names of species are indicated in parentheses after the name of each. The numbers next to some nodes represent bootstrap percentage, which are shown only for relevant nodes and those defining subfamilies (Theißen and Saedler, 1995; Theißen et al., 1996). (A) Relationship between angiosperm MADS-domain proteins. DEFH7 and DEFH68 are high-lighted by black boxes. Subfamilies are labelled with brackets at the right of the tree. The calculation is based on amino acid sequences of the MIK regions. (B) Relationship between DEFH68 and the TM3 subfamily. DEFH68 is shown in a black box. DEFH24 from *A. majus* and SOC1 from *Arabidopsis*, two MADS-box factors closely related to DEFH68 are high-lighted in open boxes. Brackets represent different plant groups indicated at the right. The calculation is based on the MIK protein sequence of the proteins using SQUA as an outgroup.

A



B

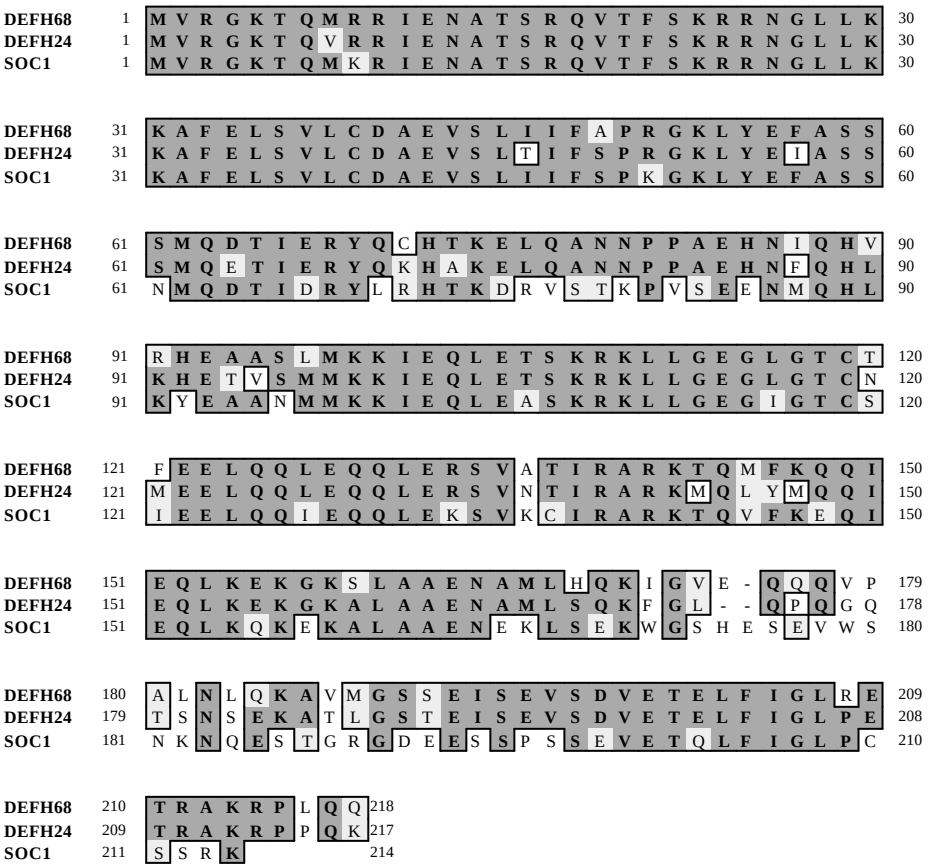


Figure 3-6. Comparisons between the predicted amino acid sequences of DEFH7 and TM8 (A) and between the predicted amino acid sequences of DEFH68 and its most closely related protein DEFH24 in *A. majus* and SOC1 in *Arabidopsis* (B). Dark and light shading indicates identical and conserved amino acids, respectively.

3.3 Expression studies with the two new *Antirrhinum* MADS-box genes

The expression patterns of *DEFH7* and *DEFH68* in different organs of wild-type *Antirrhinum* were investigated by three different techniques. Northern blot analysis was used to estimate the level of *DEFH7* and *DEFH68* transcription in poly(A)$^+$ RNA samples and, subsequently, low level of transcription was confirmed by RT-PCR which is more sensitive to detect small amounts of transcripts. In addition, *in situ* hybridization using sense and antisense probes was used to reveal the spatial and temporal expression pattern of the two genes in different organs and at different stages of development.

3.3.1 Expression analysis by Northern blot hybridisation

Northern blot analysis revealed that the *DEFH7* transcript accumulates in bracts and in inflorescences, whereby the signal in inflorescences is most likely coming from the bracts. No signal could be detected in other tissues tested (Figure 3-7). It is, therefore, likely that the *DEFH7* gene is exclusively expressed in bracts.

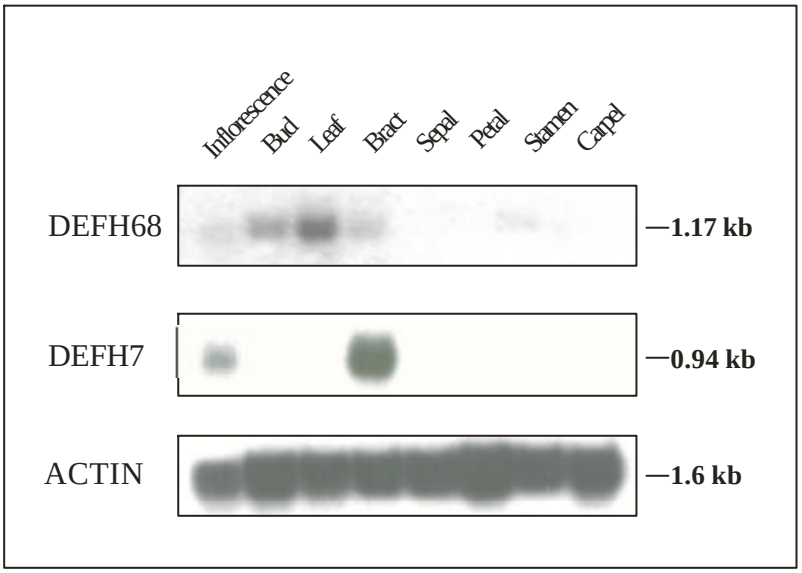


Figure 3-7. Expression of *DEFH7* and *DEFH68* in *Antirrhinum majus.* Each lane in the Northern-blots contains 2 ug poly(A)$^+$ RNA extracted from different tissues of wild type 165E plants. The filters were subsequently hybridized using the 3' end fragments of c*DEFH7* (467 bp) and c*DEFH68* (270 bp) as probes, as indicated at the left. An actin cDNA probe served as a control for equal loading of RNA samples.

Transcription of *DEFH68* was mainly detected in leaves and weakly in bracts, buds and inflorescences (Figure 3-7). A weak hybridisation signal is visible in stamens that could not

be confirmed by the more sensitive RT-PCR method (see 3.3.2). Most likely, this signal can be attributed to cross-hybridisation with *DEFH24* (see 3.2), a closely related gene that is strongly expressed in stamens (see 3.3.2).

3.3.2 Expression analysis by RT-PCR

Rare transcripts can escape detection by Northern blot analyses but can be identified by RT-PCR. For this purpose, fifty microgram of total RNA extracted from the same samples as used for the Northern blot analysis, and from roots and seedlings, was digested with DNaseI to avoid genomic DNA contamination. The RNA was then reverse transcribed, and the resulting first strand cDNA served as template for PCR analyses with gene-specific primers. The primers were designed to span introns in order to detect contamination by genomic DNA. To enhance sensitivity, RT-PCR was performed in the presence of radioactively labelled dCTP (see 2.8). Amplification of the actin cDNA in the linear phase at low cycle number served as an internal control to quantitate the PCR reactions. As shown in Fig. 3-8, all samples contained nearly equal amounts of template and no genomic DNA was amplified during RT-PCR, confirming the purity of the RNA samples.

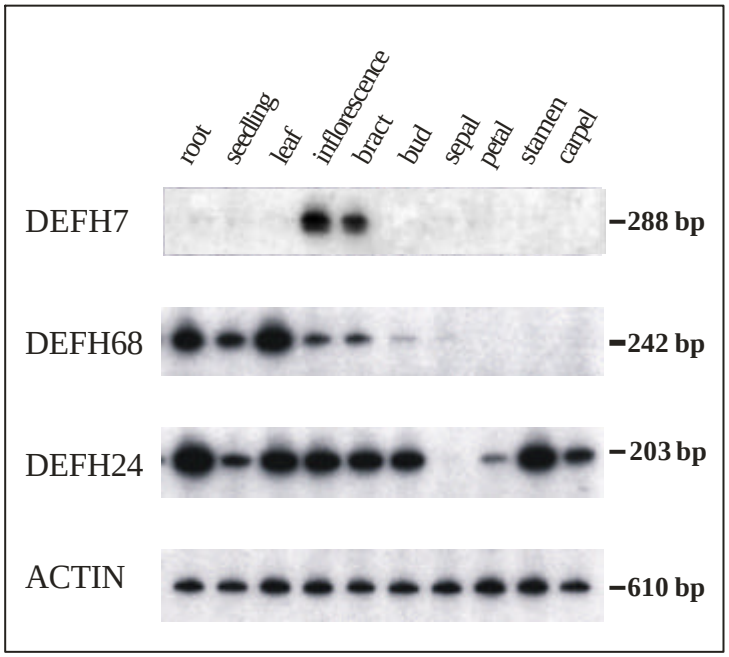


Figure 3-8. Analysis of *DEFH7*, *DEFH68* and *DEFH24* expression in various *Antirrhinum* tissues by RT-PCR. 5 ng of first strand cDNA synthesized from total RNA was used for PCR amplification with gene-specific primers (indicated at the left) in the presence of P^{32} dCTP. To control the quantity of the cDNA templates used for PCR actin was amplified at sixteen cycles. The origin of the mRNA is shown above the lanes and the sizes of the PCR fragments are indicated at the right.

In contrast to the single *DEFH7* transcript detected by Northern analysis, two bands were produced by RT-PCR (Figure 3-8). There are two possibilities: the larger and more intensive band most likely corresponds to the correctly spliced transcript, the smaller and weaker band possibly corresponds to transcripts produced by differential splicing. Inspection of the genomic sequence revealed a possible cryptic 'acceptor site' in the fourth exon, which, when used in the mRNA processing, would generate a mRNA 14 bases shorter than the normal one.

DEFH7 expression detected by RT-PCR is restricted to the bracts (Figure 3-8), as already observed by Northern blot analysis (Figure 3-7). Roots and seedlings, not investigated by Northern blot analysis, are also devoid of transcription detectable by RT-PCR. Bracts from different positions within the inflorescence and the youngest leaf beneath the first flower were harvested and examined by RT-PCR to learn about the transcriptional regulation of *DEFH7* expression during bract development (Figure 3-9). *DEFH7* is expressed in the youngest bracts tested (that is, the smallest bract shown in Fig. 3-9) and expression gradually decreases in older and larger bracts. No expression could be detected in the youngest leaves below the inflorescence and in old bracts. Taken together, the results obtained by expression studies indicate a possible role for *DEFH7* during bract development. Identification of *DEFH7* as the first bract-specific gene in *Antirrhinum* opens the possibility to study specification of these organs during development in the future.

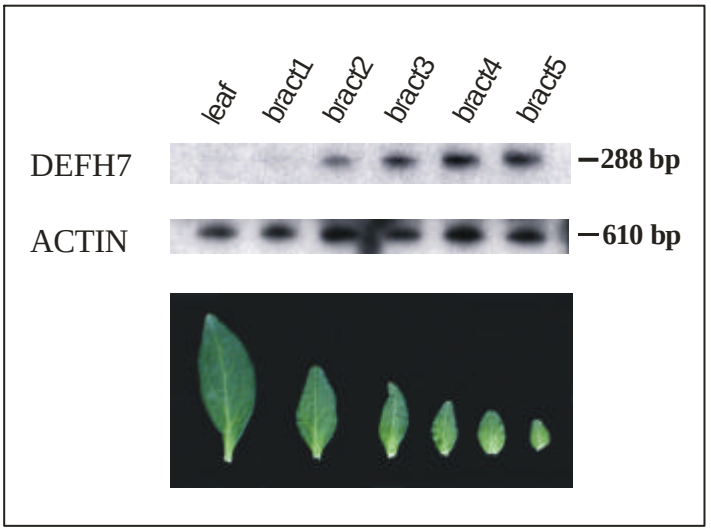


Figure 3-9. Analysis of *DEFH7* expression in leaves and bracts by RT-PCR. Bracts of different sizes and the youngest leaf beneath the inflorescence from *Antirrhinum* wild type Sippe 50 plants were collected for total RNA isolation. The length of the leaf is 2.7-3.0 cm, and bracts 1 to 5 are 1.9-2.1 cm, 1.5-1.7 cm, 1.1-1.3 cm, 0.7-0.9 cm and = 0.5 cm long, respectively. cDNAs were synthesized from these samples for PCR amplification using gene-specific primers as described in the legend to Figure 3-8. The origin of the mRNA is shown above the lanes and the sizes of the PCR fragments are at the right. All other experimental details and symbols are the same as described in the legend to Figure 3-8.

RT-PCR analysis revealed a strong signal for *DEFH68* in leaves and roots, while a weaker signal could be seen in seedlings, inflorescences and bracts (Figure 3-8). Phylogenetic ananlysis indicated that DEFH68 belongs to the same subfamily as DEFH24, suggesting that the two genes possibly share similarity of expression patterns. RT-PCR analyses with primers designed to amplify *DEFH24* were performed, using the same RNA samples as above, to test this assumption. Indeed, similar to *DEFH68*, *DEFH24* is expressed in all vegetative organs, most strongly in roots. In contrast to *DEFH68*, however, *DEFH24* is also expressed in all floral organs except for sepals. Within floral organs strongest expression was detected in stamens (Figure 3-8).

3.3.3 Expression analysis by *in situ* hybridization

The spatial pattern of *DEFH7* expression was further investigated by *in situ* hybridisation using longitudinal sections of young inflorescences. Unfortunately, no hybridization signal could be detected in developing bracts. The reason of the failure is not clear and seems likely to be related to technical problems rather than to a low level of gene expression. The Northern blot analysis (Figure 3-7) revealed a fairly high level of *DEFH7* expression in bracts that should be detectable in *in situ.*

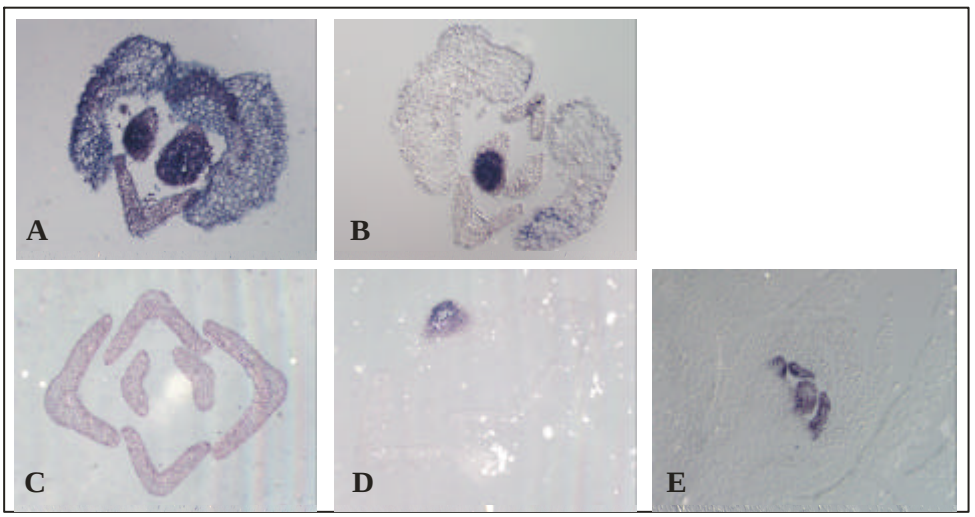


Figure 3-10. Localization of *DEFH68* mRNA by *in situ* hybridization in wild-type *Antirrhinum* plants. Serial cross sections of shoot apices were hybridized with the digoxigenin labelled sense (B and D) and antisense (A and C) *DEFH68* RNA probes. Pink to violet stained areas indicate the presence of transcript in the cells. Notice that the stain within the tip of the leaves detectable by the sense probe is due to the presence of glandular cells that frequently hybridise to single stranded probes (P. Huijser, personal comunication). Antisense *DEF* RNA probe was used in E as positive control for the whole procedure. All panels are at the same magnification.

Based on Northern blot and RT-PCR analyses, *DEFH68* is strongly expressed in leaves. To determine its site of expression in more detail, cross sections of shoot apices containing leaves at different developmental stages were hybridized with digoxigenin-labelled sense and antisense RNA probes. Strongly and uniformly distributed *DEFH68* expression could be detected in young organs (Figure 3-10A) that gradually decreased in older leaves along the shoot (Figure 3-10C).

3.4 Yeast two-hybrid screens to identify partners interacting with DEFH7 and DEFH68

Previous studies demonstrated that MADS-box proteins form specific homo- and/or heterodimers, providing the molecular basis for controlling of a variety of developmental processes (see 1.4). Detection of interaction of DEFH7 and DEFH68 with a MADS-box partner whose function is known could thus give a hint for their function and/or can help to isolate additional, previously unidentified MADS-box proteins.

The two-hybrid system provides an assay for detecting protein-protein interactions in yeast and also to screen for unknown interactors (Tucker et al., 2001). The system is based on the observation that transcription factors consist of two separable domains, responsible for DNA binding and transcriptional activation. Two different vectors, one carrying the DNA-binding domain (the bait) and the other carrying the activation domain (the prey) are used to generate fusions of the separated domains to genes encoding proteins that potentially interact with each other. If the two chimeric proteins interact, a functional transcription factor will be reconstituted and activate transcription of a reporter gene (Tucker et al., 2001). To study the network of possible interactions and, potentially, to isolate additional MADS-box proteins, yeast library screens were carried out with an *Antirrhinum* cDNA library as the prey and with DEFH7 and DEFH68 as baits.

3.4.1 Detection of partners interacting with DEFH7 and DEFH68 by a yeast two-hybrid library screen

The yeast strain Y190 carrying plasmid BD/DEFH7 or plasmid BD/DEFH68 (see 2.14.1) was transformed with a cDNA expression library cloned into the pGAD424 vector (Davies et al., 1996), and a total of 12.0 x 10^6 and 6 x 10^6 recombinant cells, respectively, were plated onto His-selective media. This initial screen identified 436 candidate colonies for DEFH7 and 631 for DEFH68.

The candidates were then tested for activation of a second reporter gene encoding the enzyme β-galactosidase (LacZ) also driven by the Gal4 promoter (see 2.14.3.1). In the case of BD/DEFH7 163 colonies out of the 436 original candidates were able to activate both reporter genes, while 70 out of 631 candidates could be validated by this test for DEFH68 (Figure 3-11 and Table 3-1).

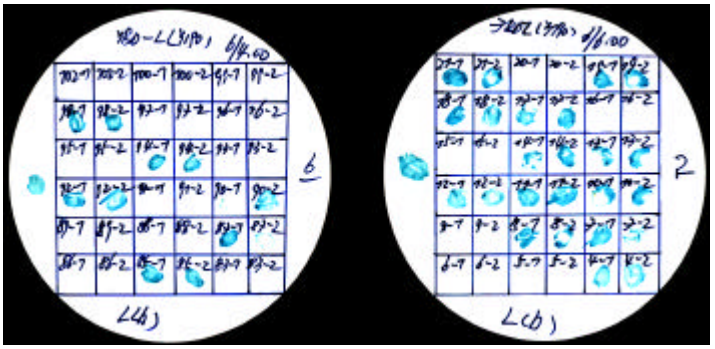


Figure 3-11. β-galactosidase (β-gal) assays for interactions with DEFH68 (at the left) and DEFH7 (at the right) detected in a yeast two hybrid library screen. The positive colonies in the first round of screen in yeast were tested twice (-1, -2). Blue color shows interactions between a bait and the prey whose colony number after selection on His - is indicated on the filter.

Table 3-1. Results of the two-hybrid screen with DEFH7 and DEFH68

Bait	Transformants screened	His $^{+}$ colonies picked	LacZ $^{+}$ colonies
Y190 (BD/DEFH7)	12.0×10^6	436	163
Y190 (BD/DEFH68)	6.0×10^6	631	70

The candidates were grouped by homology of their inserts determined by cross-hybridisation between them and the longest insert of each group was sequenced. The two largest groups (with 48 members in the case of DEFH68 and with 70 members in the case of DEFH7) corresponded to DEFH70, one of the previously identified but not characterized *Antirrhinum* MADS-box proteins (Nacken, 1990). All other groups were much smaller and none of them revealed clear homology to known proteins. Some of these groups represented interactors for both baits. The interactions with these potential partners remain to be analysed in the future. The properties of DEFH70, as a MADS-box protein and partner of both DEFH7 and DEFH68, however, have been studied further (see 3.4.3).

3.4.2 Interactions of DEFH7 and DEFH68 with known MADS-box proteins

In addition to the library screening described above, possible protein interactions between DEFH7 and DEFH68 with other known MADS-box family members in *Antirrhinum* have been tested. One new MADS-box protein was included in this analysis, AMM1, a member of the MADS-box family that is strongly expressed in leaves and weakly in bracts (Kim J. H., unpublished data).

In most of the instances no interactions could be detected, except for the interaction of DEFH7 with SQUA (Table 3-2). Interestingly, DEFH24, a close relative of DEFH68, shares the same set of interacting partner with DEFH68.

Table 3-2. Testing for interaction between known MADS factors

	DEFH7	DEFH68	DEFH70	DEFH24
DEFH7	-	-	+	-
DEFH68	-	-	+	-
DEFH70	+	+	+	+
DEFH24	-	-	+	-
PLE	-	-	+	-
SQUA	+	-	+	-
DEFH200	-	-	+	-
DEFH84	-	-	+	-
DEFH72	-	-	nt	nt
DEF	-	-	-	-
GLO	-	-	-	-
AMM1	-	-	+	-

nt = not tested

3.4.3 Characterisation of DEFH70

As revealed by the yeast library screen, DEFH70 is a dimerisation partner of both DEFH7 and DEFH68 (see 3.4.1). In fact, analysis of interactions between known MADS-box proteins revealed that DEFH70 interacts with many of the tested MADS-box proteins (Table 3-2).

A prerequisite for protein interactions in a biological context is that the partners are expressed in the same tissues. To test whether this is the case for *DEFH70*, RT-PCR analysis was performed using the same tissue samples as for the other RT-PCR experiments (see 3.3.2). *DEFH70* is expressed, as *DEFH7* and *DEFH68* are, in bracts (Figs. 3-7 and 3-8). In addition, *DEFH70*, like the vegetatively expressed genes *DEFH68*, *DEFH24* and *AMM1* included in

the protein interaction experiment, is expressed in all vegetative parts tested, mainly in roots, seedlings and young leaves. In floral organs transcript could be detected in stamens (Figure 3-12), the organ in which the floral B- and C-function genes are expressed together with *DEFH84* and *DEFH200* (Davies, 1996). The overlapping patterns of expression thus indicates that interactions between DEFH7, DEFH68 and the other MADS-box genes with DEFH70 might occur also *in vivo*.

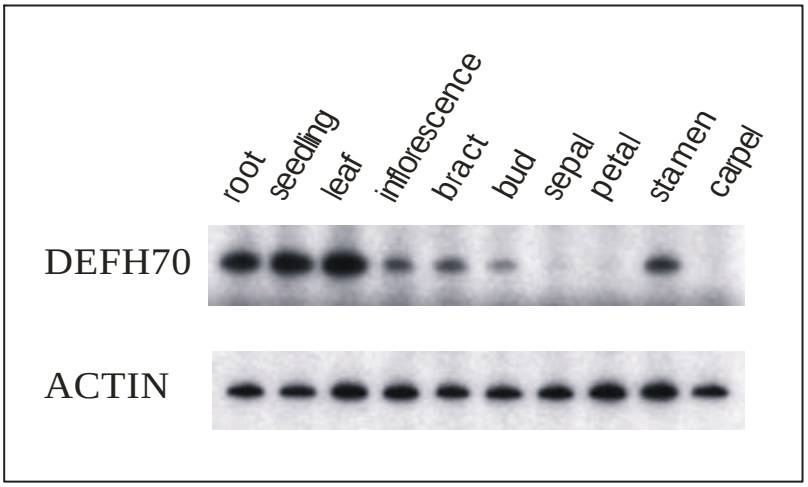


Figure 3-12. RT-PCR analysis of *DEFH70* expression in different *Antirrhinum* tissues. All experimental details and symbols are the same as described in the legend to Figure 3-8.

A possible explanation for the broad range of DEFH70 interaction partners *in vivo* could be that it provides an activation domain to protein complexes where the partners lack such a domain. To test this assumption the ability of DEFH70 to activate transcription was studied in yeast. For this purpose it was cloned into the pGBT9 vector containing the Gal4 DNA binding domain, and the resulting plasmid was transformed into the yeast strain Y190. A β-galactosidase assay with this construct showed that, indeed, DEFH70 can activate transcription of the reporter gene in the absence of the pGAD424 plamid which contains the GAL4 activation domain.

The C-terminal domain of some MADS-box proteins is assumed to contain the transcription activation domain (see 1.1.1.3). To investigate whether this is the case for DEFH70, two truncated versions of DEFH70 were generated and fused to the GAL4 DNA binding domain. One construct contained only half of the amino-terminal part of the G-domain (amino acid 1 to 205 of the proteins) and the other lacked the entire C-terminal region (amino acid 1 to 170). Both truncated proteins failed to activate the yeast reporter genes (Table 3-3), suggesting that the activation domain resides within the C-terminal domain. Interestingly, only the truncated

version lacking half the C-terminus retained the capability to interact with DEFH7 and DEFH68 in the two-hybrid experiment. This might indicate that the first half of the C-terminus is important for protein-protein interactions between DEFH70 and other proteins.

Bait \ *Prey*	DEFH70	DEFH70(Δ1/2C)	DEFH70(ΔC)	GLO	Empty vector
DEFH7	+	+	-	-	-
DEFH68	+	+	-	-	-
DEFH70(Δ1/2C)	+	+	-	-	-
DEFH70(ΔC)	+	+	-	-	-
DEF	nt	nt	nt	+	nt

Δ = deletion

nt = not tested

Table 3-3. Results of β-gal assay for the interaction of DEFH70 with DEFH7 and DEFH68. Different truncated versions of the DEFH70 protein were used as preys: in Δ1/2C half of the C-domain was deleted and in ΔC the deletion removed the entire C domain. The interaction shown between DEF and GLO is the positive control.

3.5 Searching for mutants of DEFH7 and DEFH68 by reverse genetic screening

Expression analyses with the *DEFH7* gene suggest a possible role for this MADS-box transcription factor during bract development. Isolation of a "loss of function" mutant with a phenotype showing an alteration in bract development, or even the complete loss of the bract, is an important step to prove this assumption.

Insertion of a transposon into a gene often leads to the interruption of the coding sequence and subsequently to the loss of the function of the encoded protein. Examples for such mutants are the *globosa* and *plena* mutants, both of which are caused by transposon insertion (Tröbner et al., 1992; Bradley et al., 1993). A new strategy to identify loss of function mutants takes advantage of the fact that the *Antirrhinum* genome contains several active transposons, named Tam (Transposon antirrhinum majus) elements (Carpenter et al., 1988; Sommer et al., 1988). A population of plants carrying randomly integrated Tam elements in their genomes that can serve to isolate insertion mutants of a gene of interest was established by the group of E. Coen and R. Carpenter (John Innes Centre, Norwich). The strategy of a screen for mutants in this population is as follows. Plants are screened by PCR to identify the particular insertion mutant. For this purpose primers are used that are specific for the gene of interest in combination with primers derived from the proximal or from the distal end of transposable

elements. Amplification occurs only if a transposon is inserted within the gene or within a short distance upstream or downstream of it (Figure 3-13). Since the population contains approximately 30,000 plants, the screen is carried out with pools of plants in three steps to minimize the effort of identification of insertion events in individuals. In the first screen PCR products are amplified with a gene-specific and a Tam element-specific primers on 66 pools of DNA each containing DNAs of approximately 450 plants. After hybridization with a gene-specific probe, positive pools can be confirmed in a second round of screening. The subpools of 150 plants corresponding to a positive '450 plant pool' are tested for the presence of the insert. Each positive '150 plant pool' is then subdivided into ten smaller subpools containing DNA derived from 15 plants which will be tested in the last round of screening. Seeds collected from the positive pool containing 15 plants will be sown and the seedlings are examined for the integration of a Tam element into the gene of interest. Plants carrying the insert are then selfed to obtain homozygotes for the insertion which then can be studied further.

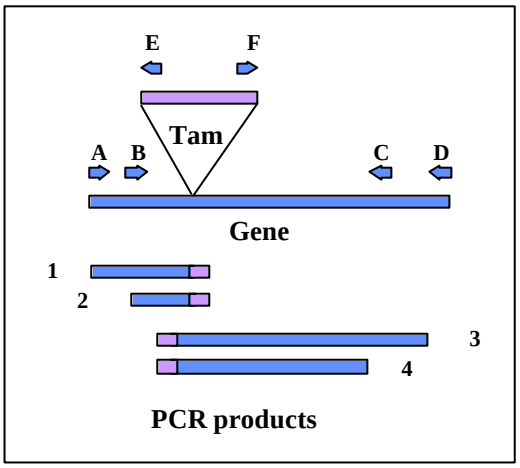


Figure 3-13. Schematic diagram of the PCR-based identification of a transposon insertion into a gene of interest. A, B, C and D indicate the position of gene specific primers at the 5'or 3' end of the gene . E and F are primers derived from the end of a transposable element of *Antirrhinum* (Tam). A polymerase chain reaction using primers A and E would lead to fragment 1, whereas the combination using the nested primer B together with primer E would amplify the shorter fragment 2. A similar result would be expected to produce fragments 3 and 4 using the primer F at the other side of the tranposon in combination with primers D and C.

To identify mutant plants for the new MADS-box genes *DEFH7* and *DEFH68*, specific oligonucletides derived from their 5' and 3' ends were designed (see 2.13.1). Transposon-specific primers were generated for various Tam elements (see 7.2). Each of the gene-specific

primers was combined with each of the transposon specific primers and used for PCR according to the strategy described above.

Figure 3-14 summarizes the results obtained searching for an integration event into the *DEFH7* genomic sequence. The combination of a *DEFH7*-specific primer with a primer common for the left (conserved) end of the transposons Tam2, Tam4, Tam5, and Tam6 amplified fragments in pools No. 20, 38 and 59 which hybridized to a DEFH7-specific probe. All three pools were identified with the same primer combination, thus the orientation of the transposable element into *DEFH7* in the putative mutant plants must be identical: the left border of the element will be orientated towards the 3' end of the gene.

In the next round of screening three subpools (A, B, C), corresponding to the positive pools of the first round, have been analyzed for the presence of the PCR product obtained in the larger pools. In all cases subpool A seemed to contain DNA derived from a plant carrying the transposon insertion into the *DEFH7* gene (Figure 3-14B). PCR using a nested primer for *DEFH7* led to the detection of the expected size reduction of the fragment for the subpools 20A and 59A (Figure 3-14B, also see strategy in Fig. 3-13). In subpool 38A a very faint band was observed with the nested primer, that could be amplified in other experiments (not shown). Since the fragment sizes detected in the three subpools are different, it can be concluded that the integration events are independent and that the putative mutant plants contain transposon integrations at different positions of the *DEFH7* gene. This is clear in Figure 14C, where the last step of the screening strategy of DNA pools is summarized. Ten subpools for each of the positive pools in Figure 3-15B (No. 20A, 38A, and 59A) were tested according to the strategy described before and always one out of ten pools gave a positive hybridization result. The positive candidate pools No. Q374, W462, and W787 (Figure 3-14C) have been confirmed by a second experiment. The site of integration was tested by determining the size of the fragment amplified between the Tam primer and the *DEFH7* 3' primer in the potential mutants in comparison to the 1730 bp long fragment, produced when using the 5' and 3' *DEFH7* primers on wild-type control DNA. According to this estimate, the integration leading to the fragment size of 1.25 kb in pool Q374 and to 1.5 kb in pool W426 must have occurred approximately at the position of the second intron of the gene, whereas in pool W787 the fragment size of about 1000 bp indicates a location within the fourth exon of the *DEFH7* gene.

Stimulated by the positive results obtained from screening the different DNA pools and the possibility to identify three different mutant alleles for the *DEFH7* gene, the progeny of the plants in pools Q374, W426, and W787 was extensively screened for individuals with the corresponding integration event. More than 500 plants have been analyzed so far, but, unfortunately, in no case could the expected mutant plant be identified.

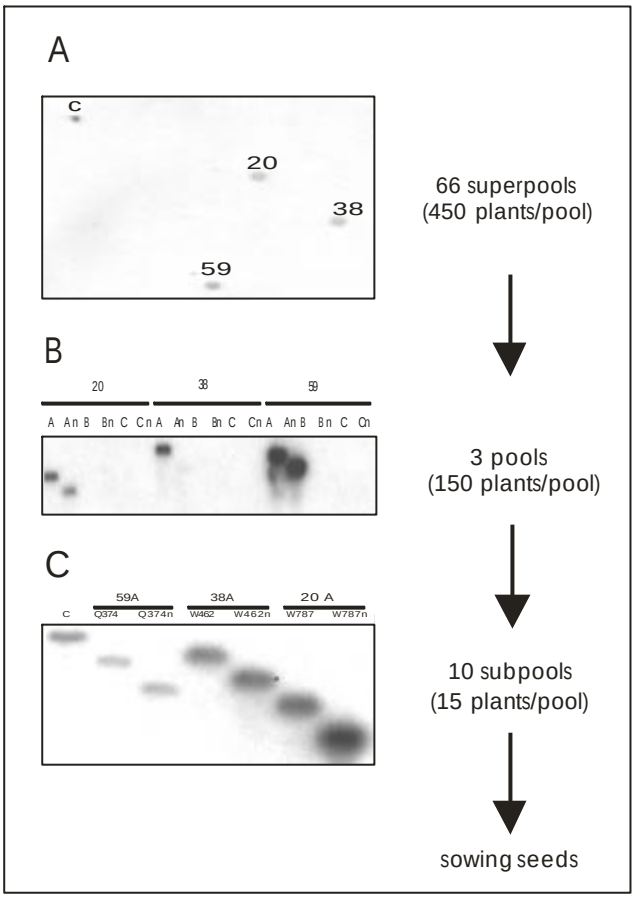


Figure 3-14. Identification of the Tam element insertion mutants for *DEFH7*. The flow chart at the right shows steps of the PCR screening process from the initial analysis of superpools to identifying single mutant plants.
(A) Dot blot with PCR products from 66 superpools of 450 plants each, hybridized with a labelled *DEFH7* probe. Three positive pools 20, 38 and 59 indicating a Tam element insertion into the *DEFH7* genomic sequence have been identified.
(B) Southern blot with PCR products obtained from the three sub-pools (A, B, and C) of 150 plants each, consistent with the corresponding 450 plant superpools. Each pool has been tested twice by using a *DEFH7*-specific primer and a nested primer (n) in combination with the same Tam element-specific primer. Subpool A turned out to be positive after hybridization to the *DEFH7* probe.
(C) Southern blot with PCR products obtained after screening 10 subpools containing 15 plants each. Only the positive subpools Q374, W462, and W787 derived from the 150 plant pools in (B) are shown here. The same nested primer (n) as in (B) was used for PCR amplification. C=positive control amplified with *DEFH7*-specific primer pairs.

The same screening strategy, using gene specific primers, was performed also for *DEFH68*, and putative insertions of a Tam transposon could be identified (data not shown). However, as

in the case of *DEFH7*, no mutant plant was obtained in the last step of screening. A possible reason for this failure is that the integration events occured somatically during growing the plant material to generate the pools. While this is likely for two of the pools containing DEFH7 mutants, the third pool was prepared in a way that excludes detection of somatic events to be identified in the last step of screen. Lethality of the mutant can not explain the absence of heterozygotes in the progeny, since heterozygotes were detected in the pools prepared from the parental lines.

In spite of the negative result at the last step of the reverse genetic screen described above, an additional mutant screen on DNA pools derived from a population of known *Antirrhinum* mutants was performed. These mutants were collected during 1900 and 1960 and described by Stubbe (Stubbe, 1966). The pools of DNA from about 400 mutants, kindly provided by R.Carpenter and R. Coen (JIC, Norwich), were generated in a way that each mutant is present in at least two different pools; one pool encoded by a number and the other by a letter, allowing to identify a mutant in a single round of PCR/hybridisation experiment. Positive hybridization results due to integration of a transposon in a gene of interest should therefore always occur in a 'number' pool and in a 'letter' pool, and the integration event in both pools should result in the same fragment size produced by PCR.

The primer combination of a DEFH7-specific oligonucleotide corresponding to the 5' end of the cDNA and the primer for the conserved end of the Tam elements 2, 4, 5, and 6, mentioned before, was used for PCR with these pools. Three 'number' pools (13, 14 and 15) and three 'letter' pools (I, L and S) gave hybridizaton signals in southern blot analysis (Figure 3-15, see next page). The fragments amplified in pool 13 and in pool L showed the same size of 0.8 kb and the size of both fragments decreased by the expected 176 bp when using a nested *DEFH7*-specific primer. According to the pooling strategy the result identified the mutant *impressa* as carrying an integration event in the *DEFH7* gene. *Impressa* mutants have smaller and more round leaves than wild-type plants and are smaller and less green (Stubbe, 1966). No phenotype related to bract morphology was described by Stubbe (1966) and could also not be observed when growing *impressa* mutant plants under greenhouse conditions; this observation was further confirmed by PCR amplification of the DNA isolated from *impressa* mutant plants where no integration in *DEFH7* could be identified.

In the other four positive pools 14, 15, I and S the lengths of the fragments produced by amplification using the DEFH7/Tam primers was identical and also the reduction of fragment

size was similar when using the nested DEFH7 primer (Figure 3-15). Therefore, all letter/number combinations (14I, 14S, 15I and 15S) were considered as putative candidates, corresponding to *Sippe2249*, the mutant *marmorea*, the mutant combination *mat hero lat elo* and *Sippe ragusa*, respectively. However, testing the DNA isolated from individual plants grown in our greenhouse from seeds obtained from the Gatersleben stock centre by PCR failed to confirm the integration event. Therefore, I conclude that the transposon insertion in the DNA pools was a recent event, which happened in the plants grown to establish the DNA pools in Norwich. Unforturnately, no seeds from these plants were available, such that I could not identify a knock out mutant of *DEFH7*.

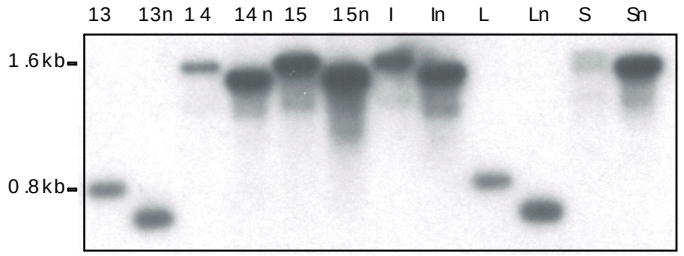


Figure 3-15. Reverse genetic screen using DNA pools from known *Antirrhinum* mutants. A southern blot is shown with six candidate pools (13, 14, 15, I, L, S.) hybridising with a *DEFH7* specific probe. Except for the pooling strategy (see RESULTS 3.5) all experimental details were the same as described in the legend to Figure 3-15.

3.6 Expression of *DEFH7* and *DEFH68* in transgenic *Arabidopsis* and *Antirrhinum* plants

Ectopic expression of genes frequently results in aberrant 'gain of function' phenotypes, that are informative for the study of their function. Alternatively, over-expression of transgenes with pronounced homology to endogenous sequences frequently results in co-suppression of the endogenous gene and in the loss of its function. In the case of MADS-box proteins that function as homo- or heterodimers, over-expression in a heterologous system can, in addition, result in a dominant 'loss of function' mutation, due to formation of non-functional protein complexes between the heterologous and endogenous proteins, thereby depleting the cells for the functional protein.

To get further information about the function of the *DEFH7* and *DEFH*68 genes by generating such mutants, constructs carrying the respective cDNAs in sense orientation with respect to the cauliflower mosaic virus (CaMV) 35S promoter were used to transform *A.*

thaliana by vacuum infiltration. For each construct 20 independent bulks of lines were obtained. In the T1 generation plants transgenic for *DEFH7* or *DEFH68* produced aerial rosettes, a phenotype that is frequently observed when plants are grown under short day conditions (Boyes et al., 2001). To observe heritable defects caused by the transgenes, plants have to be grown under controlled conditions when analysing the T2 generation in the future. Due to time restrictions these studies could not be completed during this thesis.

Agrobacterium-mediated transformation has also been used to introduce sense and antisense constructs carrying the *DEFH7* and *DEFH68* cDNAs into *Antirrhinum* (see 2.15.1). Generation of transgenic *Antirrhinum* plants is based on labour-intensive tissue culture steps. Although several transgenic calli passed the different selection media (Figure 3-16), no transgenic plant could be rescued for the constructs.

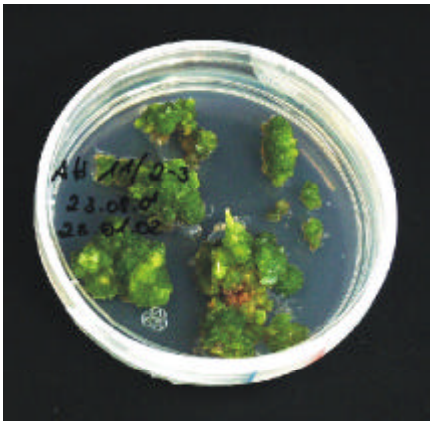


Figure 3-16. Transformed *Antirrhinum* calli growing on H5 selection medium. The calli shown here are representatives for the different constructs (35S::DEFH7as, 35S::DEFH7se, 35S::DEFH68as and 35S::DEFH68se) used for transformation.

3.7 Complementation of the *Arabidopsis* mutant *soc1* with *DEFH68* and *DEFH24*

According to the phylogenetic analysis based on the MIK region of MADS-box genes, *DEFH*68 and/or *DEFH*24 from *Antirrhinum* are the putative orthologues of the *Arabidopsis SOC*1 (*AGL*20) gene. *SOC1* is involved in regulating flowering time and mutant plants flower later than wild type. Often, orthologues have a similar function in different species. Genetic analyses have placed late-flowering mutants in at least three parallel genetic pathways based on the effect of each mutation on the response to environmental conditions. *SOC*1 integrates signals from all three pathways, which are the photoperiod, the vernalization, and the autonomous floral induction pathways (Lee et al., 2000). To assess the function of *DEFH68*

and *DEFH24* in development and to confirm whether they play a similar role as *SOC*1 in *Arabidopsis*, complementation experiments with *soc1* mutant plants were carried out using the full-length *DEFH68* and *DEFH24* cDNAs under the control of the CaMV 35S promoter. T1 plants are now growing in the greenhouse and in future generations it can be tested whether *DEFH68* and/or *DEFH24* can rescue the *soc1* late-flowering phenotype.

DISCUSSION *CHAPTER 4*

4.1 Isolation of new MADS-box transcription factors

4.1.1 Analysis of the strategy applied to isolate new MADS-box factors

Compared to screening of a cDNA library and to yeast two-hybrid screens for new MADS-box genes, a genomic library screen should facilitate isolation of all members of this family. Nevertheless, only three new MADS-box genes were identified by this strategy, although the total number of known MADS-box genes in *Antirrhinum* should be much higher (see 3.1). This result might reflect that the screening procedure was not as successful as expected. There are several possible reasons for this failure. First of all problems are very likely caused by the hybridization step. Closely related MADS-box genes share very high homology along the entire amino acid sequence, for instance, DEFH68 and DEFH24 share 82% identity which is also reflected by a 88.5% identity at the nucleotide level. Hybridization at low stringency to obtain related gene sequences and, subsequently, at high stringency to exclude already known genes, might eliminate genes like *DEFH68*, when *DEFH24* is included in the probe.

Another problem may be related to the process of grouping candidates according to the size of genomic *Rsa*I inserts. In principle, the fragment sizes obtained for different MADS-box genes should differ, because at least one of the *Rsa*I sites is most likely located within the more divergent intron or leader sequences flanking the short exon with the MADS-box. In rare cases, however, the MADS-box itself can contain two *Rsa*I sites, and restriction of genomic sequences from two closely related genes can possibly result in identical fragment sizes. Alternatively, by chance, the size of the fragments derived from different genes might be very similar. In order to avoid such problems it would be necessary to sequence all members of a group.

4.1.2 The newly isolated genes are type II MADS-box genes

All MADS-box transcription factors contain a conserved DNA-binding domain, the MADS-box. Based on this definition, four new MADS-box transcription factors from *Antirrhinum* were identified, designated DEFH7, DEFH17, DEFH20, and DEFH68. Recent studies on the phylogenetic relation between the major clades of plant MADS-box genes identified two types of MADS-box factors in plants, type I and type II (see 1.1), whose MADS-boxes contain slightly different conserved amino acids. Most plant MADS-box factors belong to the well-characterized type II proteins, classified by the conserved MIKC domain structure. In contrast, type I MADS-box factors generally lack the K-domain and are poorly understood so far

(Alvarez-Buylla et al., 2000; Svensson et al., 2000). DEFH7 and DEFH68 both encode proteins containing the type II MADS-box, followed by an I-region, a less conserved K-domain, and a most divergent C-terminal domain. Therefore, they are type II MADS-box factors (see Fig. 3-2A and 2B). Full-length cDNAs are not available for DEFH17 and DEFH20, but the presence of conserved amino acids in the MADS-box qualify them as type II MADS-box genes.

The failure to isolate cDNA clones for *DEFH17* and *DEFH20* can have several reasons. Possibly, the respective genes participate in transcriptional control of processes in a specific tissue that was not represented in the cDNA library used for the screening. Alternatively, it is possible that the abundance of the transcript is low, for instance if the genes are transiently expressed or are expressed at a very low level. *DEFH17* and *DEFH20* could also be non-transcribed pseudogenes; recent genome projects discovered a high number of pseudogenes in plant (http://mips.gsf.de/proj/thal/db/tables/tables_gen_frame.html) and human (International Human Genome Sequencing Consortium, 2001) genomes.

4.1.3 *DEFH7* reveals an unusual genomic organisation

Previous comparison of the genomic structure of MADS-box factors showed that in many cases there is a large intron immediately after the MADS-box region. For example, the first intron of *PFG* from *petunia* is about 3.0kb (Immink et al., 1999), while that of *SQUA* from *Antirrhinum* is about 4.0kb (Huijser et al., 1992). In contrast to many MADS-box factors described till now, the first intron of *DEFH7* with only 165bp is quite small. Also the B-function factor AP3 from *Arabidopsis* has a very short 101bp long first intron. Whether different sizes of the first intron is related to the function of MADS-box proteins remains unknown at present.

4.2 Expression analyses: hints for the function of the new MADS-box genes in development

Genetic and molecular studies of MADS-box factors has shown that the domain of their expression is largely similar to the domain of their function (Soltis et al., 2002), indicating that MADS-box genes are basically regulated at the transcriptional level. If this is the common feature of MADS-box factors, it raises the possibility that expression domains reflect the function of unknown MADS-box genes.

4.2.1 A potential role for *DEFH7* in bract development

Northern blot and RT-PCR analyses of *DEFH7* with RNA extracted from different *Antirrhinum* tissues showed that this gene is only expressed in bracts. This indicates that DEFH7 may play a role in bract initiation and/or development in *Antirrhinum*. In addition, RT-PCR analysis of *DEFH7* further showed differential splicing. The relevance of this is currently unknown and has to be pursued in the future. One aspect for such studies would be to determine whether there are differences in the ratio between the splicing variants in bracts at different stages of development, indicative for a role of differential splicing for the function of *DEFH7*. One could then clone the variants to see whether both of them have a protein coding capacity and, if yes, to compare their properties in an assay for protein-protein interactions, for instance, in a yeast two-hybrid assay.

Alternative splicing was also observed in other MADS-box genes. For instance, tissue-specific alternatively spliced ZEMa forms were present together with ubiquitously distributed transcripts. Alternative splicing further increased the possibility for ZEMa proteins to form variant heterodimers with other MADS-box proteins (Montag et al., 1995). Whether the alternative splicing of DEFH7 generates different protein products having different functions in wild type *Antirrhinum* remains unclear.

4.2.2 *DEFH68* and *DEFH24* in vegetative development

Similar MADS-box genes participate in similar developmental processes in different plant species. For instance, the B function genes *DEF* and *GLO* specify petal and stamen development in *Antirrhinum* (Sommer et al., 1990; Tröbner et al., 1992), as do *AP3* and *PI* in *Arabidopsis*, *OsMADS4* in *rice* and *SIL1* in *maize*. For *DEFH68* and *DEFH24*, two structurally related MADS-box genes (see 1.2.3.2), Northern blot and RT-PCR analyses indicated that they share similar expression patterns in vegetative tissues and different patterns in floral organs (Figure 3-8); *in situ* hybridization further showed that *DEFH68* and *DEFH24* both are strongly expressed in leaf primordia (Göttlich 1992), and that the intensity declined gradually for *DEFH68* in older leaves. Decrease of expression might be caused by decay of mRNA or by a down-regulation of gene expression in aging organs, perhaps driven by a senescence program or by some other mechanisms.

4.3 Phylogenetic analysis of MADS-box genes in plants

4.3.1 *DEFH7* and *TM8* constitute a new subfamily of MADS-box genes

Previous studies on the plant MADS-box gene family suggested that the *Lycopersicon* gene *TM8* represents an 'orphan group', meaning that orthologues had not been identified from other angiosperm species (Purugganan et al., 1995; Theissen et al., 1996; Purugganan, 1998). Phylogenetic reconstruction for *DEFH7* revealed that *DEFH7* and *TM8* constitute a new subfamily, termed the TM8 subfamily, suggesting that the *DEFH7* gene is a *TM8* orthologue from *Antirrhinum*.

For orthologues, the primary hypothesis would be that they share similar expression patterns and functions (Theißen 2002). Surprisingly, however, the genes under discussion here have quite different expression patterns: the *DEFH7* gene is specifically expressed in bracts, and the transcripts of *TM8* accumulate in the inner three floral organs, i.e. petals, stamens and carpels (Pnueli et al., 1991). Previous studies have shown that for most MADS-box genes, the expression domain is coincident with the domain of function (Soltis et al., 2002). The clear differences in the expression patterns of *DEFH7* and *TM8* thus strongly suggest that these genes play different biological roles in plant development. If so, in at least one of the lineages that led to the extant genes a change in gene function must have occurred – an interesting event for orthologues genes which deserves further studies.

Another large difference between these two putative orthologues is that the TM8 protein has an unusually short C-terminus containing only 20 amino acids. Whether this basic structural difference might influence the molecular mode of TM8 action remains unknown. A molecular analysis in *Brassica oleracea* var. *botrytis* has shown that the cauliflower phenotype is due to a C-terminal mutation, which is caused by a stop codon generated in exon five and resulting in a 150 amino acid protein product instead of a wild type protein of 255 amino acids; this indicates that the C domain is required for protein function despite its highest rate of evolutionary change. The rapid evolution of the sequence outside of the DNA-binding domain of SRY, which is a mammalian sex determination gene, was also observed to affect its function; it may be associated with speciation (Whitfield et al., 1993).

Furthermore, the divergent and rapidly evolved C domain is also important to stabilize the formation of the ternary complex between the heterodimers of DEF-GLO and a homodimers of SQUA-SQUA, indicating that the C domain is important for multiple protein interactions (Egea-Cortines et al., 1999). Therefore, it might be interesting to investigate whether TM8 and DEFH7, respectively, also form ternary complexes with other factors, and how the constituents interact with each other.

Whether the basic structure difference between DEFH7 and TM8 is correlated with morphological evolution remains an open question. Recent work does indicate that sequence changes, including loss of a C-terminal domain, may indeed be associated with morphological variation (Omland, 1997; Galant and Carroll, 2002; Ronshaugen et al., 2002). It will be interesting to see, therefore, whether this also holds for TM8-like genes.

In order to uncover the origin causing different protein structures and expression patterns between DEFH7 and TM8 and the relationship between the morphological change and molecular variation in DEFH7 and TM8 genes that regulate different developmental processes, it will be interesting to characterize orthologues from other species like the close *Antirrhinum* relative *Linaria*, test their protein structures and expression patterns as to whether they are more closely related to *DEFH7* or *TM8*, and estimate the divergence time among subfamily members. This analysis might dissect possible links between molecular and morphological diversity, and further explain why there is no *TM8* orthologue available in the fully sequenced genome of *Arabidopsis*. Is this corresponding to the fact that *Arabidopsis* has no bracts?

4.3.2 DEFH68 is a member of the TM3 subfamily

TM3 subfamily members share similar expression patterns except for AGL14 from *Arabidopsis*, and it has been speculated that they may also share highly conserved functions (Theissen et al., 1996). Another MADS-box gene from *Antirrhinum*, *DEFH24*, is also a member of this subfamily, and forms a monophyletic clade with *DEFH68* with 100% bootstrap support. Both proteins are identical for 82% along their entire length. Such high sequence similarity with the combination of the overlapping expression patterns (see results) may indicate that they duplicated recently and have a redundant function; in addition, *DEFH24* is also expressed in the floral organs petals, stamens and carpels. This may indicate that DEFH24 has additional functions in flower development.

Functional redundancy is not unusual within the MADS-box gene family. *SHP1* and *SHP2* MADS-box genes in *Arabidopsis* share 87% identity at the protein level and show almost identical expression patterns in the *Arabidopsis* fruit. Neither single mutant shows a detectable phenotype; in contrast, *shp1 shp2* double mutants have a dramatic phenotype: the mature fruits fail to dehisce (Liljegren et al., 2000). This result strongly suggests that *SHP1* and *SHP2* share a redundant function controlling fruit dehiscence in *Arabidopsis*. It may be necessary,

therefore, to obtain mutants for both *DEFH24* and *DEFH68*, in order to uncover their developmental function in *Antirrhinum*.

The phylogenetic tree further suggests that *SOC1* from *Arabidopsis* is an orthologue for both of them. *SOC1* is a key regulator of flowering time; it integrates signals from three independent floral induction pathways. Analysis of its expression has shown that the strongest expression is observed in leaves, but expression is also detected in vegetative apices, inflorescence, and stems of flowering plants and roots (Lee et al., 2000). Therefore, *SOC1*, *DEFH68* and *DEFH24* share similar expression patterns (see 3.3.2). In order to explore whether *DEFH68* and *DEFH24* play roles as flowering time genes in *Antirrhinum* development in a similar way as *SOC1* does, overexpression of both *DEFH24* and *DEFH68* in wild type *Arabidopsis* and complementation experiments on *soc1* mutants are currently being performed. Respective results might provide important information about the biological roles of these genes.

4.3.3 Phylogenetic analysis of DEFH17 and DEFH20

Phylogenetic reconstruction based on the MADS-box region alone usually indicates correct gene relationships, at least at the gene subfamily level (Theißen et al., 1996). Phylogenetic trees established with representative angiosperm MADS-box genes show that *DEFH17* belongs to the *AGL17* subfamily (data not shown). *DEFH125* from *Antirrhinum* is also a member of this subfamily. According to the expression data obtained so far, the subfamily members have divergent expression patterns. But the members from the same species have similar expression patterns: AGL17, AGL21 and ANR1 are all expressed in roots. Along this line of reasoning, *DEFH17* may also be involved in pollen development, as *DEFH125* does. In order to test this assumption it is important to get a full-length cDNA. This hypothesis might also explain why a full-length cDNA clone could not be identified by a total plant cDNA library screening, because RNA from pollen certainly does not make up a larger fraction there. It might be worth to screen a library containing different stages of pollen development.

The phylogenetic tree also shows that *DEFH20* does not belong to any known subfamily (e.g., the hypothesis of a close relationship to the AG subfamily has only 26% bootstrap support, and *DEFH20* is on a long branch, suggesting that its position is due to an artifact known as "long branch attraction" (data not shown)). In addition, the DEFH20 MADS-domain shows some strange sequence deviations that have not been found so far in known plant MADS-

domains, such as a valine at position 4 and two isoleucines at positions 46 and 47. These data suggest that *DEFH20* represents a new subfamily for which other members have yet to be found.

4.4 Protein-protein interactions

It is becoming clear that a major mechanism underlying transcriptional control in eukaryotic cells is the formation of multi-protein complexes which results in networks to regulate multiple targets (Marcotte et al., 1999; Riechmann and Ratcliffe, 2000; Schwikowski et al., 2000). One of the primary methodologies that allowed for the analysis of direct protein interactions is the yeast two-hybrid system.

4.4.1 DEFH68 and DEFH24 share common interacting partners

Expression pattern comparison between *DEFH68* and *DEFH24* and phylogenetic analysis suggests that the two genes may share redundant functions in specifying vegetative organs of *Antirrhinum*, and late in flower development DEFH24 might be involved in the control of development of floral organs as it is expressed in petals, stamens and carpels. These observations, together with data indicating that MADS-box factors typically interact with other proteins (Riechmann and Ratcliffe, 2000), suggested that DEFH68 and DEFH24 may share a common set of protein interactors, and that additional proteins may interact specifically with DEFH24. Using the yeast two-hybrid assay, DEFH70 interacts with both DEFH24 and DEFH68. The function of DEFH70 has yet to be determined, but analyses of their expression patterns (see 3.4.3) showed a partial overlap to DEFH68 and DEFH24 indicating that such interactions can occur in vivo.

There were also non-MADS-box proteins interacting with DEFH68 in the yeast two-hybrid screening (see 3.4.1), confirming that MADS-box proteins not only interact with family members but also interact with other different factors (Davies et al., 1999; Gamboa et al., 2001). It will be very interesting to explore whether DEFH24 interacts with these proteins.

Interestingly, DEFH68 and DEFH24 do not interact with each other. Nevertheless, they possibly interact indirectly by forming multi-protein complexes with a common set of proteins or they may function in parallel to regulate vegetative development.

As mentioned above, DEFH24 might have another set of interactors to specify floral development. The protein interaction assay for DEFH24 in yeast, however, showed that it does

not interact with the A-, B-, or C-function proteins involved in the control of floral organ identity (see 1.2.3.1). There are several possibilities to explain how DEFH24 might participate in the control of flower development. Firstly, it could be involved in organ identity control by interacting with the ABC function MADS proteins, provided that such interactions are mediated by other proteins. Such ternary complexes would not be detectable in the yeast two-hybrid assay. Second, DEFH24, might interact with factors other than floral organ identity MADS proteins to contribute to the control of flower development.

4.4.2 Interactors of DEFH7

Interestingly, direct interaction was observed between DEFH7 and the floral meristem identity controlling protein SQUA in yeast. Expression of these genes overlaps in the bracts and this suggests that the interaction between the two proteins may occur in vivo in the control of bract development. In fact, expression of DEFH7 in bracts of *squa* mutant plants is decreased compared to wild-type (data not shown), indicating that DEFH7 transcription partly depends on SQUA activity and hence could be controlled by a DEFH7/SQUA heterodimer in an autoregulatory manner. In *squa* mutants inflorescences are composed of normal-looking bracts and in their axils new inflorescences develop instead of flowers in a reiterated manner (Huijser et al., 1992). Reduced expression of *DEFH7* in *squa* bracts might then indicate that the organs are not fully determined as bracts. Sometimes, however, flowers can develop in *squa* mutants suggesting redundance of the SQUA function. One can speculate, that the redundant proteins interact with DEFH7 and that, in absence of SQUA this protein complex controls DEFH7 transcription. Elevated DEFH7 expression then allows bract formation and flower development in the *squa* mutant.

4.4.3 Interactions of DEFH70 with other MADS-box proteins

DEFH70 interacts with at least nine different MADS-box factors with five different types of temporal expression patterns and all these patterns overlap with that of DEFH70. This indicates a potential *in vivo* relevance of these interactions.

Interestingly, DEFH70 also interacts with the class C protein PLE. Expression of DEFH70 overlaps with that of PLE in stamens, whose identity is controlled by the C function (see 1.2.3.2). It is possible, therefore, that the PLE/DEFH70 dimer is involved in the control of some aspects of stamen development. Stamen identity, however, is also controlled by the B-function proteins. In *Arabidopsis*, a ternary complex is formed between the B proteins AP3,

PI, the C protein AG and SEP3 (see 1.2.3.2). In *Antirrhinum*, such tetramer can possibly form between DEFH70, PLE and the class B proteins DEF and GLO. A ternary factor assay in yeast can confirm this possibility in the future.

Alternatively, multiple interactions can occur between DEFH70 and different classes of MADS-box factors implying that DEFH70 may play important roles in *Antirrhinum* development, and is possibly required for different development aspects by differential interactions between different MADS-box factors.

4.4.4 Implications of complex protein networks

Multiple heterodimerization among the same family members of transcription factors is a common biological process in specifying cell fate. Previous data and the results obtained here suggest that members of the plant MADS-box family, a large group of transcription factors, can selectively heterodimerize and control different aspects of plant development. DEFH68, DEFH24 and DEFH7 share the interaction partner DEFH70, but only DEFH7 interacts with SQUA. DEFH70, however, a protein whose transcript is present in all the vegetative organs tested, as well as in bracts and stamens, has almost no restrictions to form protein-complexes with many MADS-box proteins with different temporal expression patterns. Taken together, these results suggest that a large variety of MADS-box factors coexist in a specific type of cells at certain stages of development, some of which preferentially form multiple heterodimers and can thereby determine differentiation of a particular cell. In addition, other factors such as relative DNA-binding affinity and specificity of the complex, complex formation with other non-MADS box factors, and binding site selection may also participate in a specific regulatory program to control plant development.

With the completion of many genome sequences, functional genomic studies are of great importance in characterizing proteins that have been newly discovered by genome projects. The graphical representations of protein-interaction maps in yeast demonstrated the complexity of protein associations in cellular processes (Schwikowski et al., 2000; Tucker et al., 2001). 1548 out of 2709 published yeast protein interactions could be linked in a single large network and the functional category of unknown proteins can often be predicted by direct and indirect interactions with known partners. It is estimated that the average number of interactions would be about 4.5 to 5.8 in yeast and 5 in C. elegans (Tucker et al., 2001). It is clear from the studies on the large-scale of a fraction of all protein interactions in yeast that the

intricate behavior of a cell is much more complex than can be exhibited by a two-dimensional interaction map. In the near future, results gained from studies of small genomes, such as that of *S. cerevisiae*, will provide a basis for future exploration of more complex, multicellular organisms.

The well-characterized mammalian and yeast MADS-box factors SRF and MCM1 form protein-protein interactions with a variety of other proteins (Shore and Sharrocks 1995). Recently there was one report on the characterization of an in vitro interaction between the plant MADS-box protein AG in *Arabidopsis* and non-MADS factors, a Leucine-rich repeat factor FLOR1 and an acid phosphatase protein VSP1 complex (Gamboa et al., 2001). This evidence further supports the idea that the plant MADS-box factors might interact with other proteins (see 3.4.1). Based on the accumulated knowledge, it appears that the genome-wide protein-protein interaction maps have to be built, in order to understand comprehensive MADS-box protein activities in plant cells.

Coping with the emerging data sets, many techniques to detect protein-protein interactions have been developed in mammalian and plant systems. One advanced tool to directly visualize protein interactions in plant cells was developed recently. This method is based on fluorescence resonance energy transfer (FRET). The MADS box proteins were fused to either cyan fluorescence protein or yellow fluorescence protein and transiently expressed in protoplasts. FRET spectral imaging microscopy and FRET-fluorescence lifetime imaging microscopy measurements were performed on those protoplasts to detect protein-protein interactions in planta (Immink et al., 2002).

4.6 Outlook

Determination of the function of a trandcription factor is a main challenge with the increasing number of genes available by numerous approaches. A comprehensive understanding of the biological function of a protein will require many information on different levels such as the knowledge of transcriptional, translational and posttranslational regulation, DNA-binding properties, three dimensional structures, and the interplay of the protein with other molecules in a cell.

As a first step towards uncovering possible functions of the two MADS-box genes *DEFH7* and *DEFH68*, expression analyses and phylogenetic reconstruction have been performed. Protein-protein interaction assays in yeast have identified several interaction partners for both

of them. In order to identify the function of these genes more precisely, loss-of-function and gain-of-function mutants have yet to be obtained. However, despite intensive studies, only a few MADS-box genes in *Antirrhinum* have been associated with a loss-of-function phenotype by reverse genetic means, and most of the progress in the characterization of the MADS family in *Antirrhinum* has focused on MADS-box genes controlling flower development. A number of genes such as *DEFH68*, *DEFH24* and *DEFH70* are also expressed in vegetative parts, suggesting that MADS factors also play important roles beyond flower development in *Antirrhinum*.

Much need to be learned about the regulatory machinery mediated by the MADS-box factors; only few downstream target genes of MADS factors have been identified, little is known about the types of complexes formed by these proteins, how these combinatorial complexes change DNA-binding affinity or specificity and how the differences in structures influence their biological roles. Homodimers, heterodimers, and multimeric complexes are able to form, but the functional importance of these complexes is not clear at present.

Therefore, there is considerable work to be done to unravel the functions of the MADS-box factors in plant development. In *Antirrhinum* studies toward this goal will begin with identification of the entity of MADS-box genes followed by identification of the genes that they regulate. Extensive DNA-microarray experiments, mapping protein-protein interactions and characterization of mutants for all MADS-box genes will be the necessary steps to achieve this goal, supported by information gained from studying other species. Such comparative studies shall provide important insights into developmental mechanisms common to all plants.

Summary

In plants, MADS-box transcription factors play a central role in many aspects of development. They have been shown to be involved in control of organ and meristem identity, root architecture, flowering time and regulation of fruit development. The MADS-box family has been defined on the basis of primary sequence similarity amongst numerous proteins from a diverse range of eukaryotic organisms including yeast, plants, insects, amphibians, and mammals. The MADS-box is a contiguous conserved sequence of 56 amino acids constituting the DNA-binding domain of these proteins. The name refers to four of the originally identified members: MCM1 (from yeast), AG and DEF (from plants), and SRF (from humans).

In this dissertation the isolation of new members of this family from *Antirrhinum majus* by genomic library screening is described. These new MADS genes are named *DEFH7*, *DEFH17*, *DEFH20*, and *DEFH68* (*DEFICIENS-HOMOLOGUE*). The expression studies by Northern blot and RT-PCR analyses show that *DEFH7* is only expressed in bracts, and *DEFH68* mainly in vegetative parts of the plant. To identify the functions of these two new MADS-box factors, reverse genetic screens were carried out to obtain insertion mutants of these genes possibly displaying an altered phenotype which could hint to their roles in *Antirrhinum* development. Furthermore, sense and antisense constructs of cDNAs were introduced in wild type *Antirrhinum* and sense constructs in *Arabidopsis* plants. Since the phylogenetic analyses indicate that the SOC1 in *Arabidopsis* might be the orthologue of either DEFH68 or DEFH24, another known MADS-box factor, from *Antirrhinum*, complementation experiments using DEFH68 and DEFH24 were performed in the *Arabidopsis* mutant *soc1*. The analysis of transgenic plants is underway.

Heterodimerization of transcription factors is a common feature which has been postulated to enhance their regulatory potential. To understand the network of protein-protein interactions of MADS-box transcription factors in *Antirrhinum majus*, yeast two-hybrid screening was performed to identify interactors for both DEFH7 and DEFH68. True interactors were first confirmed by β-galactoside (β-gal) assay and by immunoprecipitation experiments for further examination of the interactions.

Zusammenfassung

MADS-box Proteine spielen eine zentrale Rolle in vielen Aspekten der Entwicklung von Pflanzen. Es wurde gezeigt, daß sie an der Kontrolle der Organ- und Meristem-Identität, des Blühzeitpunktes, der Wurzelarchitektur und der Regulation der Fruchtreife beteiligt sind. Die Familie der MADS-box Transkriptionsfaktoren wurde auf der Basis von Sequenzähnlichkeiten in der Primärstruktur der Proteine definiert und beinhaltet Proteine aus verschiedenen eukaryotischen Organismen wie Hefen, Pflanzen, Insekten, Amphibien und Säugetiere. Die als MADS-box bezeichnete Region besteht aus 56 hoch konservierten Aminosäuren, welche für die Bindung der Proteine an DNA verantwortlich sind. Der Name leitet sich aus den Anfangsbuchstaben der vier zuerst beschriebenen Mitglieder der Familie ab: MCM1 (aus Hefe), AG und DEF (aus Pflanze) und SRF (vom Mensch).

In dieser Arbeit wird die Isolierung neuer Mitglieder dieser Familie von Transkriptionsfaktoren aus *Antirrhinum majus* mittels Durchmusterung einer genomischen Bibliothek beschrieben: *DEFH7*, *DEFH17*, *DEFH20*, and *DEFH68 (DEFICIENS-HOMOLOGUE*). Die Analyse der Expression mittels Northern und RT-PCR Technologie zeigt, daß das *DEFH7* Transkript ausschließlich in den Bracteen nachweisbar ist, während das *DEFH68* Transkript vorwiegend im vegetativen Teil der *Antirrhinum* Pflanze detektiert wird. Hinweise auf die Funktion der durch die beiden Gene kodierten Proteine sollte anhand von Mutanten bestimmt werden. Hierzu wurde eine Löwenmäulchen-Population durchmustert, welche zufallsmäßige Integrationen von mobilen Elementen, sogenannten Transposonen, im Genom hat. Außerdem wurden transgene Pflanzen in *Antirrhinum* und *Arabidopsis thaliana* erzeugt, welche entweder “Sense” oder “Antisense”-Sequenzen der Gene überexpremieren. Phylogenetische Analysen ergaben, daß SOC1 aus *Arabidopsis* ein mögliches orthologes Protein für DEFH68 bzw. DEFH24 sein könnte. Aus diesem Grund wurde die *soc1* Mutante zwecks Komplementationsanalyse mit Konstrukten der beiden *Antirrhinum*-Gene transformiert. Die Analyse dieser Pflanzen konnte im Rahmen dieser Arbeit nicht mehr abgeschlossen werden.

Eine oft beschriebene Eigenschaft von Transkriptionsfaktoren ist deren Heterodimerisierung, wodurch deren regulatorisches Potential erhöht wird. Um das Netzwerk an Protein-Protein Interaktionen von MADS-box Transkriptionsfaktoren in *Antirrhinum majus* besser zu verstehen, wurde für DEFH7 und DEFH68 “Two-Hybrid” Analysen im Hefesystem durchgeführt. Interaktoren wurden sowohl mittels ß-Galaktosidase-Expressionstests als auch Immunopräzipitationsexperimenten untersucht.

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7-1 Genomic sequence of *DEFH7*

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   1 AGTAGAGTTG AAGAGAATTG AGAATCCGAC AAACAGACAA GTGACGTTTT CAAAGAGAAG AAATGGCTTG CTAAAGAAAG CTTTTGAATT TCTGTACTT
 101 TGTGATGCTG AGGTTGCTGT TCTTATCTTC TCTCCTTCAG GAAAGGCATA TCAGTATGCA AGTCATGAGT AAGTGCATGA AATTATTGAT TTTACTATG
 201 CCTTCAATCA GTTATCATCG ATCTTATTCC TTGTTCGTTT CTATTTCTTT TGAAAGTTAG ATGTACCTTG TGACAGAGAC TTATTAAGCG TGTGTAGCT
 301 AGACTCAATT TATGTTTGAT TATGGTATAA CAGCACGCAT AGGACAATTG CAAGGTATAA AAGTGAAGTT GGAATAACCA AACCAGGTGA CAGGGCATC
 401 ACATCCATGG AGGTATTCAT TTGGTGCAAA CTGAATATTA TCATCAATAT ATGTCGACGT TGAAGAAAAG AGGTTTCTCC ATGTGAATTG CATAAATAC
 501 TTGTCACTTT TAAATGTGCA GGTTTGGAGA AATGAAATTG AAGACTTAAA AAGAACTGTT GATGCCCTGG AAGCAAGAGA TATGTGTGTG TCATGCCAG
 601 AATATTTTAC ATTTTTCATG ACTAATATTT ATGTCGCCTT CTAAATTCTA TTACTTGTAA CAGGCATTTT GCTGGAGAAA ACTTATCAGG TTAGGCATG
 701 AAAGACCCTT AAACAGTTAG AACGGCAGAT AAGAATTGGG GTGGAACGTA TTCGCTCTAA AAAGGTAATT CTCATTAATT GTATTTTTGT TTGATCACT
 801 GATAATAACG TAAACATCTA TCTATCTGGT CATCGGCAGA GGCGTATCAT CGCAGAACAC ATGACTTATC TGAAGAAAAG GGTAAGTTTC CAAACTAAT
 901 ATTTTCATAT TAGAAATTAC AAATTTGAAA AATAGTGCCT AAACAATGAG TGTATGCTAT TGCAGCATAA AGACCTACAA GAAGAGAACA CAATCTCCA
1001 AAAGAGAGTG AGCATTTTAA TCAAGATTGG CACTTATTAT TGTGCTTTGT TTTTGTCTGA ATCACTCATA TATCTAAAGT ACCAATATTA TTTTGACCT
1101 GTTTGAGGTC CAAGCTACAT GAAGTTCAAG AGGCCAACAC AAGCTGCTCA ATCATTTATG ACTCAGATGG AACCAGGGTA TTCCCAGGGT ATAATAAAT
1201 AGCACAATCT GTTCATTAAC ATCCCATATG TAATCAAGAA TACGCTTCAT GCCATTATCT TATGAAGAAA TTTCAGCTTC AAATGAATGA TTAGATAAT
1301 TTACTCCTCA TTTTCTCGGT GTTTCTGTGT CTACATAGGT TTTCTTGGAC CTGTTTCAAG GACAACCATT AATCATGTAG GGTTATCTTT GCTTGATCT
1401 TGTATATTTT TCACCGTACA AGCTCCTTTT ACTACTAAAT CTTGTTGTAT TATTATTTTG TAGGATTCAA TAAATCTTCA TCAAGGATGG GGGCTGTTT
1501 TTCAATGTTA TCTGAAGATT TGCCTGGAAA AATAAAATAG CTTGCTGATC TCTCTTTTTC GTAGCCTTTC TTCAGTTTTA CTTCTTTTTA TAGATATGG
1601 ACTGCAA
```

7.2 Tam-specific oligonucleotides used in reverse genetic analysis

```
Tam 1 r:       5'- TCTTGGGACATAGGTTTTATGCGACAGTT - 3'
Tam 2.4.5.6 l: 5'- TCTTGGGACATAGGTTTTATGCGACAGAT - 3'
Tam 3 r:       5'- ACGGCTCGGCACGTTTACCATCTT - 3'
Tam 3 l:       5'- AATTGGCACGGCCCAATTCACATCTT - 3'
Tam 7 r:       5'- GGTTTCGTGTATTGTGACGATAATA - 3'
Tam 8 l:       5'- AAAAGTGTCCCTAACTTATTGGGACACGATTAT - 3'
Tam 9 l:       5'- AAAAGTGTCTCAAATCTATTGCGACACAACTAGAT - 3'
```

Erklärung

Ich versichere, daß ich die von mir vorgelegte Dissertation selbständig angefertigt, die benutzten Quellen und Hilfsmittel vollständig angegeben und die Stellen der Arbeit, einschließlich Tabellen, Karten und Abbildungen, die anderen Werken im Wortlaut oder dem Sinn nach entnommen sind, in jedem Einzelfall als Entlehnung kenntlich gemacht habe; daß diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; daß sie – abgesehen von unten angegebenen Teilpublikationen – noch nicht veröffentlicht worden ist sowie, daß ich eine solche Veröffentlichung vor Abschluß des Promotionsverfahrens nicht vornehmen werde.

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