MicroRNA-mediated control of floral homeotic functions in Antirrhinum: characterization of the *FISTULATA* gene

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1. INTRODUCTION

1.1 The genetic control of flower development

The transition from vegetative to reproductive growth is a critical developmental event that affects the identity of the shoot apical meristem and results in the establishment of the inflorescence meristem that subsequently generates the floral meristem. It is a complex process, governed by a cross-talk between pathways triggered or influenced by various environmental and endogenous factors (Bernier and Perilleux, 2005). In Antirrhinum this transition generates an inflorescence meristem producing leaf-like bracts in a spiral order instead of leaves in decussate phyllotaxis, with flowers instead of shoot primordia in their axils (Figure 1.1). The floral meristem then initiates floral organs in a whorled order.

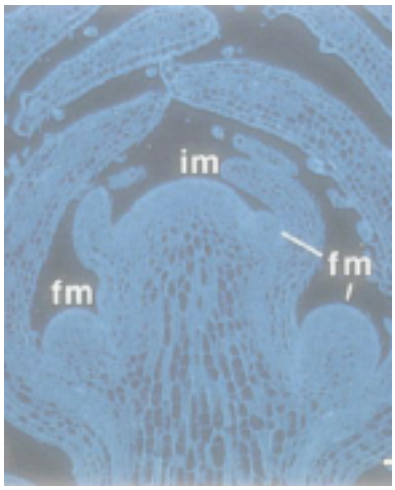


Figure 1.1: Longitudinal section of an Antirrhinum inflorescence. Im: inflorescence mersistem, fm: floral meristem produced in the axils of leaf-like bracts. From Huijser et al., 1992.

The four different types of floral organs of a wild-type flower are arranged in four whorls. In the first (outermost) whorl five sepals develop followed by five petals in the second whorl, together constituting the perianth. The third and fourth whorls contain the reproductive organs: four stamens and the staminodium in the third

whorl and two fused carpels forming the gynoecium in the fourth. After initiation of carpel primordia the floral meristem becomes determinate.

According to recent insights (Davies et al., 2006; Litt, 2007) floral organ identity is controlled by the activity of three developmental functions (Figure 1.2):

1) The meristem identity genes define as part of the (A) function the identity of the floral meristem and are responsible for sepal initiation.

2) Inside the sepal whorl the B and C organ identity functions control alone and in combination the identity of floral organs (Coen and Meyerowitz, 1991; Schwarz-Sommer et al., 1990; Weigel and Meyerowitz, 1994).

3) The cadastral function, also incorporated in the (A) function, establishes and maintains the expression boundaries of the organ identity genes.

These three functions are discussed in the following sections with major emphasis on the control of the expression domains of the organ identity genes in Antirrhinum.

1.1.1 Floral meristem identity as part of the (A)-function

When a meristem is produced in the axil of a bract, its floral identity is determined by the meristem identity genes *FLORICAULA* (*FLO*) (Coen et al., 1990) and *SQUAMOSA* (*SQUA*) (Huijser et al., 1992). Mutants of these genes are impaired in the inflorescence to floral transition and display inflorescences instead of floral characters by the reiterated initiation of bracts rather than sepal primordia. Early in development, *FLO* and *SQUA* are expressed throughout the flower meristem and, in analogy to their Arabidopsis orthologs *LEAFY* (*LFY*) and *APETALA1* (*AP1*) (Mandel et al., 1992; Weigel et al., 1992), they are likely to be involved in the activation of the B- and C- functions and at least FLO in the cadastral control of the C-function (McSteen et al., 1998; Motte et al., 1998).

The involvement of the A-function in the control of petal identity when combined with the B-function, as emphasized by the textbook ABC model (Figure 1.2 top), has been discussed in detail elsewhere (Davies et al., 2006; Litt, 2007). Importantly, in its new definition the (A)-function is responsible for determining sepal identity, which is the ground state of floral organ identity (Figure 1.2 middle panel).

1.1.2 The floral organ identity genes

The B- and C- organ identity functions are responsible alone and in combination for the development of petals (B alone), stamens (B and C together) and carpels (C alone) in their respective whorls. These functions have been defined by analyzing mutants with very similar homeotic phenotypes in Arabidopsis and Antirrhinum (Coen and Meyerowitz, 1991; Schwarz-Sommer et al., 1990; Weigel et al., 1992). The B- and C-functions are governed by class B- and class C-homeotic genes, which are transcription factors and belong to the MADS-box gene family (Schwarz-Sommer et al., 1990; Sommer et al., 1990; Yanofsky et al., 1990).

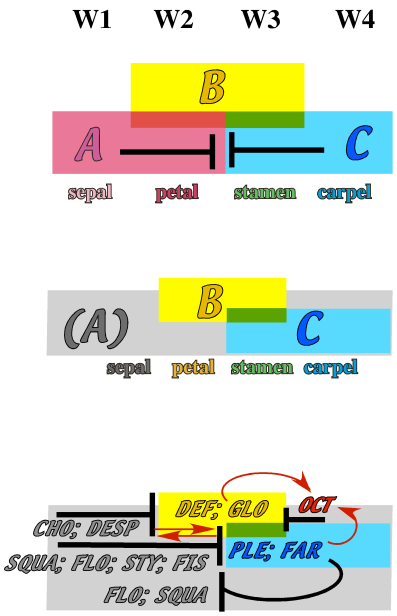


Figure 1.2: Combinatorial models for the control of floral organ identity by homeotic functions. The textbook ABC model (Coen and Meyerowitz, 1991; Weigel et al., 1992) is shown in the top panel. Three classes of genes control the identity of organs in the four floral whorls (W): sepals (A) in W1; petals (A+B) in W2; stamens (B+C) in W3; carpels (C) in W4. Overlapping colors (pink + yellow = red and blue + yellow = green) represent overlapping functions in the second and third whorl. Black barred lines indicate mutual exclusion of the A- and C- functions from the respective expression domains. In the middle panel the (A)BC model as described in (Davies et al., 2006; Schwarz-Sommer et al., 1990) is presented. The (A)-function in grey is the ground state of the floral organ identity, which is necessary for the activation of B- and C- functions and also for the control of their expression boundaries. The B- (yellow) and C- (blue) expression domains overlap in the third whorl (green). Antirrhinum genes specifying the (A)-, B- and C- functions are indicated in the panel at the bottom. Arrows indicate activation and barred lines represent repression. From Davies et al., 2006.

In Antirrhinum, mutants of the class B genes *DEFICIENS* (*DEF*) and *GLOBOSA* (*GLO*) (Carpenter and Coen, 1990; Sommer et al., 1990; Tröbner et al., 1992) show homeotic petal-to-sepal and stamen-to-carpel conversions (Figure 1.3).

Likewise, mutants of the *PLENA* (*PLE*) gene (Bradley et al., 1993) show conversion of stamens to petals and carpels to sepaloid organs and it is thus considered a C-function gene. In addition, *ple* mutants are indeterminate as revealed by initiation of a new flower inside of the fourth whorl due to reestablishment of the (A)-function in the absence of C (Davies et al., 1999). The structural relation of *FARINELLI* (*FAR*) (Davies et al., 1999) with *PLE* and its Arabidopsis ortholog *AGAMOUS* (*AG*), (Bowman et al., 1989; Yanofsky et al., 1990) defines *FAR* as the second class C-gene in Antirrhinum, although *far* mutants do not show homeotic defects.

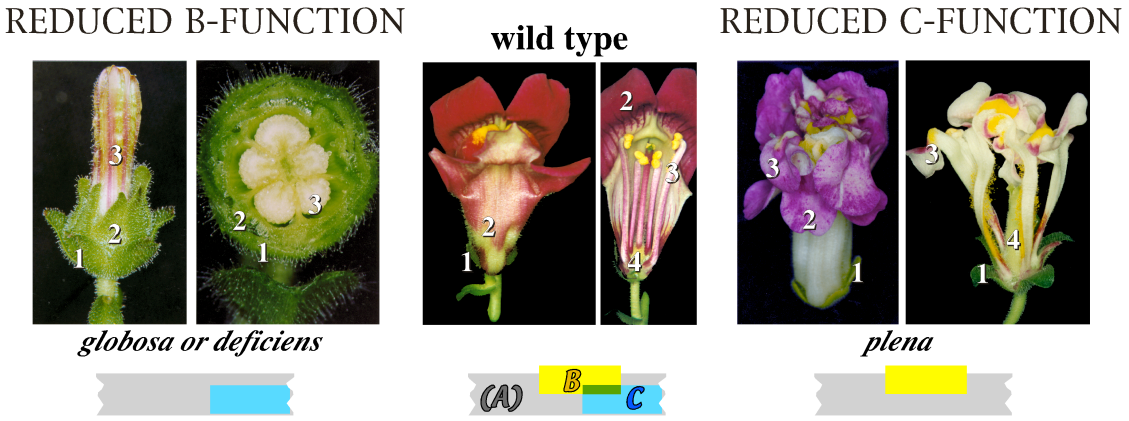


Figure 1.3: Antirrhinum mutants defective in the homeotic control of floral organ identity. The wild type flower is depicted in the middle flanked left and right by mutants in the B- and C-functions, respectively. The name of the mutants is indicated under the photographs. Whorls are numbered. The scheme beneath the photographs represents the homeotic alterations of the mutants in the context of the (A)BC model. From Davies et al., 2006.

According to their role in the specification of all floral organs as well as in the control of floral meristem identity the E-function governed by the *SEPALLATA* MADS-box genes (*SEP*) (Ditta et al., 2004; Pelaz et al., 2000) has been added to the floral ABCs (Theissen and Saedler, 2001). In Antirrhinum the E-function is performed by the so-called "intermediate" or Im-proteins, which can form dimers or higher order protein complexes with the B- and C- proteins (Davies et al., 1996; Egea-Cortines et al., 1999). Class E- genes are expressed after the meristem identity genes and before the organ identity genes and fulfill all requirements to be incorporated in the newly defined (A)- function (Davies et al., 2006).

1.1.3 The cadastral control

The domains of B- and C- gene expression are restricted to the whorls where they control organ identity. Genes that are involved in the establishment and maintenance of the domains are called cadastral genes. Mutation in cadastral genes give rise to mutant flowers showing homeotic defects due to ectopic expression of organ identity genes (Figure 1.4).

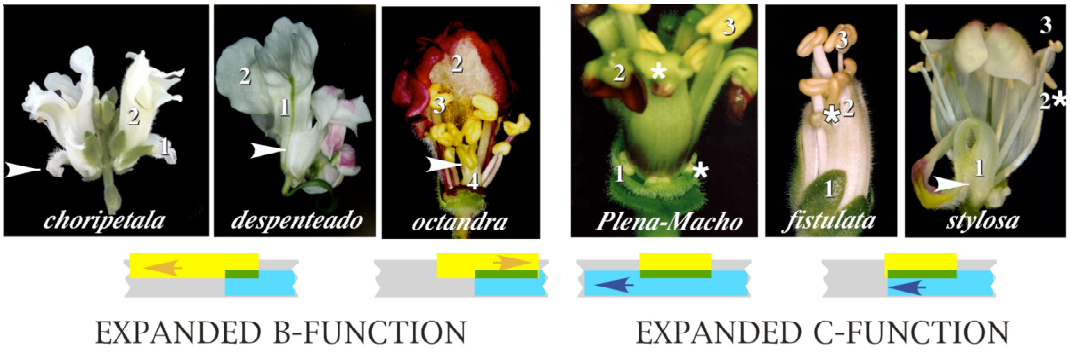


Figure 1.4: Antirrhinum mutants with expanded B- (left panel) and C- (right panel) expression domain. The name of each mutant is indicated under the photographs. Arrows point to homeotic defects due to expansion of the B-domain in the first whorl (cho and des) and in the fourth whorl (oct), while asterisks indicate homeotic defects due to expansion of the C- domain in the outer whorls. Whorls are numbered. The block scheme beneath the photographs shows organ identity alterations in the context of the (A)BC model. From Davies et al., 2006.

The first gene with cadastral function, *APETALA2* (*AP2*), has been identified in Arabidopsis, where it controls floral meristem identity as well as the outer boundaries of the C-domain (Bowman et al., 1989; Weigel, 1995). Intriguingly, *AP2* orthologs in Petunia (Maes et al., 1999) and Antirrhinum (Keck et al., 2003) do not play a role in flower development comparable to that of *AP2* in Arabidopsis, suggesting that other genes perform this function.

1.1.3.1 Antirrhinum mutants defective in the control of the B-domain

Mutants of *CHORIPETALA* (*CHO*) and *DESPENTEADO* (*DES*) show impaired control of the outer boundaries of the B-domain in that *cho* and *des* flowers exhibit some degree of petaloidy of the sepals due to ectopic class B-gene expression (Figures 1.2 and 1.4). Interestingly, *CHO* and *DES* also influence the establishment and spatial restriction of the C-domain as revealed in certain double mutant combinations (Figure 1.5), suggesting some common aspects in the B- and C-boundary control (Wilkinson et al., 2000).

OCTANDRA (*OCT*) is the Antirrhinum ortholog of *SUPERMAN* in Arabidopsis and controls the inner boundary of the B-function (Figures 1.2 and 1.4) by controlling proliferation of B-expressing cells (Davies et al., 1999).

1.1.3.2 Antirrhinum mutants defective in the control of the C-domain

Ple-macho (Lönnig and Saedler, 1994; Schwarz-Sommer et al., 1990) and *Ple*-ovu (Bradley et al., 1993) are semi-dominant alleles of *PLENA*, where ectopic C-expression in the two outer whorls is the result of a Tam3 transposon insertion in the second large intron of the *PLE* gene. The precise molecular mechanism resulting in de-repression of *PLE* is not clear, because Tam3 insertions in *PLE* oriented opposite to that found in *Ple*-macho and *Ple*-ovu result in recessive *ple* mutations (Bradley et al., 1993).

STYLOSA (*STY*) (Motte et al., 1998; Navarro et al., 2004) and *FISTULATA* (*FIS*) (McSteen et al., 1998; Motte et al., 1998) are responsible for the regulation of the C-expression domain in the inner whorls (Figures 1.2 and 1.4): *sty* and *fis* mutants show partial petal-to-stamen conversion. This defect is enhanced in the *sty fis* double mutant combination suggesting that *STY* and *FIS* interact, or act redundantly in the regulation of the *PLE* and *FAR* expression domains (Motte et al., 1998).

STY has been recently molecularly characterized (Navarro et al., 2004) being the ortholog of *LEUNING* (*LUG*) (Conner and Liu, 2000) in Arabidopsis. Based on their structural similarity to GRO/TUP1-like proteins, LUG and STY most likely act as transcriptional co-repressors lacking a DNA-binding domain (Conner and Liu, 2000; Navarro et al., 2004). *STY* and *LUG* are expressed in the inflorescence meristems and throughout young floral primordia. The ubiquitous expression patterns is thus in apparent contrast to a spatially restricted function to exclude AG/PLE from the outer whorls of the flower. Interaction between LUG and its protein partner SEUSS (SEU) (Conner and Liu, 2000; Franks et al., 2002) with AP1 and SEP3, confers repressor activity in all floral whorls in early stages of flower development (Sridhar et al., 2006). Later in development, this activity is enhanced in the second whorl by other repressing factors such as AP2 (Bowman et al., 1991; Jofuku et al., 1994) and BELLRINGER (Bao et al., 2004b), and is weakened in the inner whorls by factors which promote the C-function by inducing AG expression like LFY/WUS (Busch et al., 1999; Lohmann et al., 2001) and

AG/SEP3 autoregulation (Castillejo et al., 2005; Gomez-Mena et al., 2005). Thus, the extent of the repression by LUG/SEU in the centre of the flower is directly regulated by AG itself in that AG recruits SEP3 and excludes AP1 from the functional protein complex (Castillejo et al., 2005; Sridhar et al., 2006)

In Antirrhinum STY interacts both genetically and at the protein level with *GRAMINIFOLIA* (*GRAM*) revealed by synergistic floral homeotic defects and by yeast interaction assays (Navarro et al., 2004). The influence of *GRAM* on the C-boundary is minor, but it becomes enhanced in a double mutant combination with *sty*. Additionally, *gram* enhances the homeotic defects of several other mutants influencing the B/C boundaries such as *fis* and *cho*. *Fis* and *cho* also enhance each other's defect as well as that of *sty* suggesting that the control of the boundaries of the B- and C- expression domains is rather complex and depends on the function of several factors (Figure 1.5). The defects observed in mutants for the cadastral function are partly overcome by combination with mutants of the *FIMBRIATA* (*FIM*) gene (Simon et al., 1994). FIM encodes an F-box protein that is likely to be involved in targeting repressors of the B- and C-function for proteolytic degradation (Ingram et al., 1997). Epistasis of *cho*, *des*, *sty* (Wilkinson et al., 2000) and *fis* to *fim* (3.1.5-3.1.6) suggests that *FIS*, *STY*, *CHO*, *DES* are direct or indirect targets of this degradation.

Molecular characterization of *FISTULATA* is the goal of this thesis. A gene-to-gene comparison with known Arabidopsis repressors of the homeotic C-function was not successful suggesting that FIS performs a function that is different from that in Arabidopsis. Such differences comparing the species are also indicated by the lack of a comparable function of *AP2* orthologs as mentioned above.

Mutants in the *BLIND* gene in Petunia shows flowers with homeotic defects similar to *fis*. Accordingly, the *bl* mutation leads to ectopic expression of the class C-genes *FBP6* and *pMADS3* (Angenent et al., 1994; Tsuchimoto et al., 1993) suggesting that *FIS* and *BL* are functionally related. As described in the Results section (3.3-3.4) we indeed found that *FIS* and *BL* encode a microRNA (miRNA) related to the *MIR169* gene family.

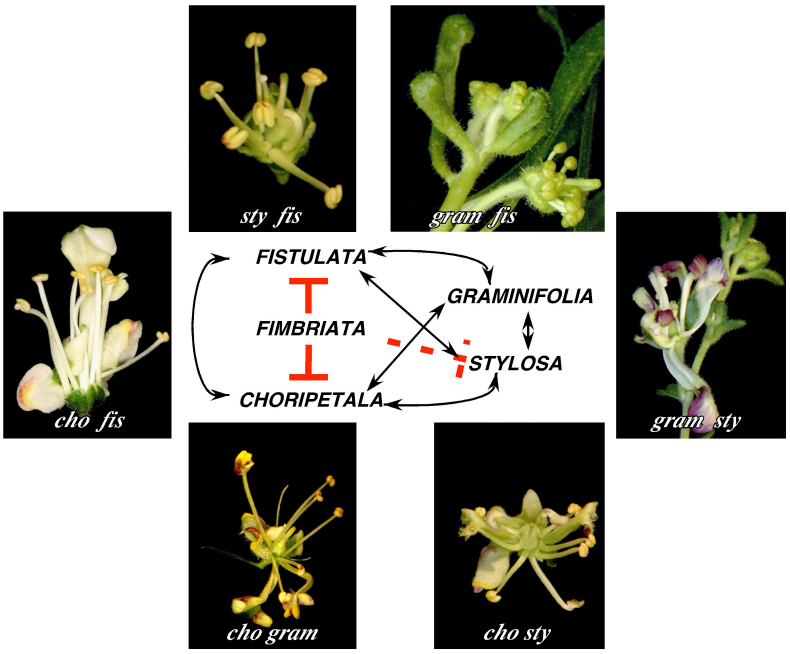


Figure 1.5: Genetic interaction between genes controlling the expression boundaries of the B- and C- domain. The double mutants (shown in the photographs) show a more aberrant phenotype as compared to the single mutants (Fig. 1.4). The genetic interactions discussed in the text are shown schematically in the diagram in the middle where black arrows indicate positive interactions and red barred lines represent negative interference. *FISTULATA*, *CHORIPETALA*, *GRAMINIFOLIA* and *STYLOSA* are abbreviated as *fis, cho*, *gram* and *sty*, respectively. From Davies et al., 2006.

As an introduction to miRNA-related topics I will briefly describe in the next section transcription processing and mode of action of miRNAs and the influence they have on gene regulation during development.

1.2 MicroRNAs in gene regulation and development

The emergence of multicellular organisms was accompanied by the generation of complex regulatory mechanisms of gene expression in order to modulate protein expression in the cell. There are many steps in the pathway leading from DNA to protein, and all of them can in principle be regulated. Thus a cell can control the proteins it makes by (1) controlling when and how often a given gene is transcribed (transcriptional control), (2) controlling how the RNA transcript is spliced or processed (RNA processing control), (3) selecting which completed mRNAs in the cell nucleus are exported to the cytosol and determining where they are localized (RNA transport and localization control), (4) selecting which mRNAs

in the cytoplasm are translated by ribosomes (translational control), (5) selectively destabilizing certain mRNA molecules in the cytoplasm (mRNA degradation control), or (6) selectively activating, inactivating, degrading, or compartmentalizing specific protein molecules after they have been made (post-translational control) (Figure 1.6). In a network of cooperating factors the precise and reliable regulation of gene expression is necessary to establish the proper temporal and spatial specification of a certain cell type and of a certain organ.

MicroRNAs are recently discovered regulatory components in this network, first identified in the *lin-4* and *let-7* mutants of *C. elegans* (Lee et al., 1993; Reinhart et al., 2000) where mutation in the respective genes lead to defects in the temporal progression of developmental events. After that many other microRNAs were found in humans, worms, flies (Lagos-Quintana et al., 2001; Lau et al., 2001; Lee and Ambros, 2001) and also in various plant species (Billoud et al., 2005; Jones-Rhoades and Bartel, 2004; Reinhart et al., 2002; Wang et al., 2004; Zhang et al., 2006) interfering with various biologically relevant processes.

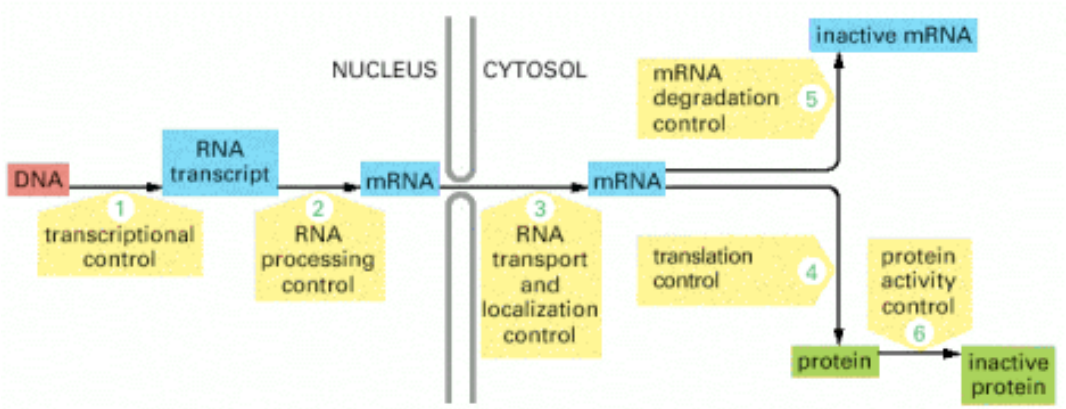


Figure 1.6: Schematic diagram of the mechanisms controlling gene expression from mRNA transcription to protein synthesis (numbered from 1 to 6). From Alberts et al., 2002.

1.2.1 MicroRNA biogenesis

Gene regulation can be influenced by different classes of small RNAs, which are non-translated, single stranded ~19-25 nucleotides (nt) long RNA molecules (Bartel, 2004) involved in the post-transcriptional regulation of target genes. They are produced from double stranded 'stem-and-loop' precursors in both plants and animals and bind to target nucleic acids by complementary base pairing. Based on

differences in their biogenesis and action, small RNAs with regulatory functions in gene expression have been grouped into different classes, the most prominent being short interfering (si) RNAs and micro (mi) RNA. In the following sections I will only consider the biogenesis and mode of action of miRNAs.

Plant miRNAs are often organized in gene families: the core miRNA sequence itself is conserved between family members while the stem-and-loop sequence can vary considerably. The 117 Arabidopsis *MIRNA* genes identified up to now give rise to 73 distinct mature microRNAs and the expression of 62 of them is experimentally confirmed. The sequences of the genes are documented in miRBASE at http://microrna.sanger.ac.uk where miRNAs of the same family but from different precursors are normally termed a, b, etc.

The three fundamental steps of miRNA biogenesis are summarized in Figure 1.7.

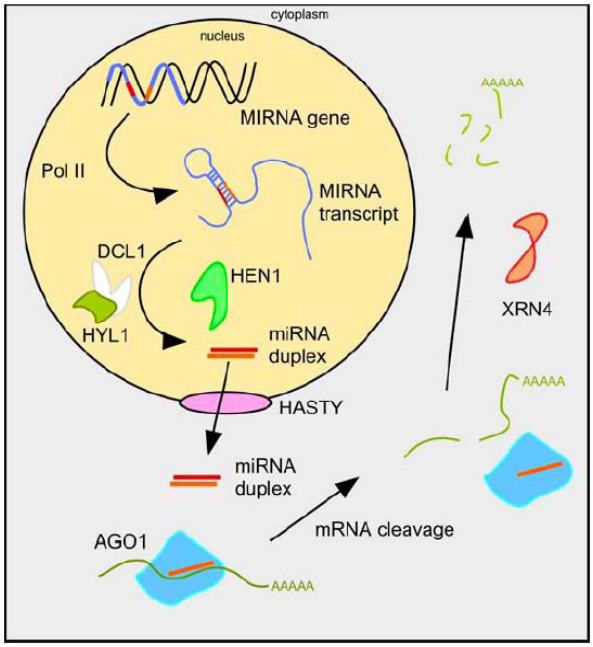


Figure 1.7: Diagram showing miRNA processing steps in plants. The primary transcript (pri-miRNA) is produced by RNA polymerase II (polII) in the nucleus and processed to the miRNA precursor (pre-miRNA) and miRNA:miRNA* duplex by DICERLIKE1 (DCL1). The duplex is then methylated by HUA ENHANCER 1 (HEN1) and exported in the cytoplasm by HASTY. In the cytoplasm the miRNA strand of the miRNA:miRNA* duplex is recruited by the RISC complex, whose major component is ARGONAUTE1 (AGO1), where it directs cleavage (or translational inhibition) of the target mRNA. From Kidner and Martienssen, 2005.

- **transcription:** Plant *MIRNA* genes are usually located in intragenic regions. They can be organized in polycistronic clusters suggesting transcription of multiple primary microRNAs from a single primary transcript (pri-miRNA) (Reinhart et al., 2002). *MIR* genes are probably

transcribed by the RNA polII enzyme resulting in a pri-miRNA transcript containing 5' guanosine caps and 3' poly-adenosine tails, as do conventional protein-coding transcripts. Furthermore, many promoter elements include TATA boxes (Xie et al., 2005).

- **maturation:** The first step is the nuclear cleavage of the pri-miRNA, which liberates a ~100 nt long stem-and-loop intermediate, known as the miRNA precursor, or the pre-miRNA (Lee et al., 2002)**.** In *Arabidopsis*, the nuclear Dicer family member DCL1 (RNAse III family) is thought to mediate both cleavage steps to form the miRNA-containing short double stranded miRNA:miRNA* duplex. These products typically contain 2 nucleotide overhangs at their 3' ends and free 5' phosphates (Elbashir et al., 2001). The *Arabidopsis* methyl-transferase HEN1 has been shown to add methyl groups to the 3' terminal ribose at both strands of the miRNA:miRNA* duplex, which prevents addition of one or several uridyl residues, to stabilize miRNAs (Yu et al., 2005). HASTY, the plant exportin-5 homolog has been implicated in shuttling miRNAs into the cytoplasm, where they can exert their function (Bollman et al., 2003).
- **incorporation in the RISC complex:** Following cleavage and nucleo-cytoplasmic export, the miRNA strands with lower thermodynamic stability at their 5' end are preferentially stabilized to perform their function in gene silencing (Khvorova et al., 2003; Schwarz et al., 2003) once incorporated into a ribonucleoprotein complex (RISC). The AGO proteins are the core component of RISC-like complexes and specifically binds small RNAs with their PAZ domains, while a second functional region, the PIWI domain is implicated in target RNA cleavage. The miRNA is directly transferred to the AGO PAZ domain, whereas the miRNA* passenger strands are selectively degraded (Cerutti et al., 2000; Hammond et al., 2001).

1.2.2 Origin and conservation of *MIRNA* genes

Most of the miRNAs identified to date are conserved among higher plants, e.g. Arabidopsis, Populus, rice and maize (Lu et al., 2005; Reinhart et al., 2002; Sunkar et al., 2005a; Sunkar et al., 2005b; Zhang et al., 2006) and also in basal plants (Axtell and Bartel, 2005). Several additional miRNA families are conserved only within specific phylogenetic lineages; miR403 is present in the eudicots

Arabidopsis and Populus but absent from the monocot Oryza (Sunkar et al., 2005a) and three families identified by cloning in Oryza are conserved in other monocots such as maize, but are not evident in the sequenced eudicots (Sunkar et al., 2005b). Within each family, the mature miRNA is always located on the same arm of the stem loop (5' or 3'), as would be expected if genes share common ancestry. Although the sequence of the mature miRNA and, to a lesser extent, the segment on the opposite arm of the hairpin to which it pairs, are highly conserved between members of a miRNA family (both within and between species) the sequence, secondary structure and length can highly diverge between members. Similarly conserved between species is the ability to regulate the expression of the same target gene family.

The fact that miRNA families regulate homologous mRNAs in basal and higher plants that have very different reproductive structures and leaf morphology suggests that microRNAs are part of a very ancient mechanism underlying seemingly different developmental outcomes (Axtell and Bartel, 2005; Floyd and Bowman, 2004).

MIRNA gene families can originate from inverted duplications in a head-to-head or tail-to-tail orientation of a complete or partial gene sequence supported by finding long stretches of homology between newly originated microRNA families (miR161 and miR163) and their target genes (e.g. the founder gene) (Allen et al., 2004). This duplication may include the promoter of the founder gene or the capture of a new promoter. Inverted duplication of the founder gene results then in the formation of hairpins that are eventually processed by the RNA machinery leading to small RNAs that can target the transcript of the original gene. Large scale duplication events together with chromosome rearrangement may then have played a role in the establishment of *MIRNA* gene families (Maher et al., 2006) suggesting that they evolved like protein coding genes.

Some gene families are characterized by a large number of members and it has been speculated that the resulting redundancy is necessary to increase the miRNA dosage necessary for target repression (Axtell and Bartel, 2005).

1.2.3 Mode of action

Plant microRNA families regulate expression of one or more members of a target gene family. The degree of complementarity between plant microRNAs and the MicroRNA Recognition Element (MRE) (Kiriakidou et al., 2004) in their target is usually very high. MREs can be found either in the coding region or in the 3' UTR of targets (Reinhart et al., 2002; Rhoades et al., 2002). Plant microRNAs can regulate their target either by regulation of transcription or by post-translational modification, e.g. mRNA cleavage or translation inhibition.

- **Transcriptional regulation:** Only one case has been reported where miRNA165/166 regulates the methylation state of the *PHABOLUTA* (*PHB*) gene by recruitment of a chromatin modifying complex at the *PHB* locus during transcription (Bao et al., 2004a). Most likely, the miRNAs recognize newly synthesized and processed PHB transcript, perhaps before the RNA is released from the template chromosome.
- **Target cleavage:** This is the best understood mode of action exerted by small RNAs to regulate gene expression. The small silencing RNAs guide the AGO component of RISC to cleave a single phosphodiester bond within complementary RNA molecules. The cleavage fragments are then released, freeing the RISC to recognize and cleave another transcript (Dugas and Bartel, 2004).
- **Translational interference:** This mechanism is not well understood but potential clues came from finding AGO proteins and miRNA targets localized to cytoplasmic foci known as Processing bodies (P-bodies) which are sites for storage and degradation of mRNAs (Liu et al., 1995). This suggests that miRNA binding directs the target to the P-bodies where they become sequestered from the translation machinery and destabilized. Examples of translation inhibition have been reported for miR172 controlling AP2 in the inner whorls of the Arabidopsis flower to prevent ectopic AG expression and for miR156/157 in the regulation of *SPL3* to prevent early flowering (Chen, 2004; Gandikota et al., 2007).

1.2.4 MicroRNAs in plant development

Plant microRNAs preferentially target transcription factors and play a central role in the control of gene regulatory networks. They are required in many developmental processes like organ polarity determination, meristem function, floral patterning, vascular development, lateral development and hormone response (Chen and Meister, 2005; Chen, 2005; Dugas and Bartel, 2004; Filipowicz, 2005; Jover-Gil et al., 2005; Kidner and Martienssen, 2005).

Two possible models explain their role in regulation of gene expression:

1) ***"clearance model":*** **miRNAs pattern the expression of the target.** If miRNAs and their targets have complementary expression domains, then the miRNAs can clear cells from transcripts transmitted by cell lineage transport after mitosis (Rhoades et al., 2002). In this way rapid cell fate transitions and differentiation of cell lineages are established. In plants, the miRNA166 expression pattern, for example, spatially defines the expression domain of the maize HD-ZIPIII family member Rolled Leaf 1 (Juarez et al., 2004).

2) ***"Fine-tuning model":*** **miRNAs control the expression level of pre-patterned genes.** MiRNAs can act as an additional layer of gene regulation by preventing fluctuation in transcript abundance during specific developmental events. The expression domain of CUP SHAPE COTYLEDON1 (CUC1) and CUC2, for example, overlaps with that of miR164 in the inflorescence, suggesting that miRNAs function in the control of the mRNA levels of the targets contributing therefore to developmental robustness (Baker et al., 2005; Sieber et al., 2007). The discovery of feed-back loops between the target and the microRNA to control the homeostasis of the target mRNA supports this model (Mallory and Vaucheret, 2006; Vaucheret et al., 2006). The stability of miR168 is, for instance, determined by the abundance of AGO1 (miR168 target) creating a feed-back loop by which AGO1 can ensure a constant expression level (Vaucheret et al., 2006) that otherwise would lead to pleiotropic developmental defects (Mallory and Vaucheret, 2006).

1.3 *MIR169* and its targets: the *NF-YA* transcription factor family

As mentioned before, we found that *FISTULATA* is related to the *MIR169* gene family which has been first identified in Arabidopsis (Reinhart et al., 2002). It represents one of the largest microRNA families with 14 members in Arabidopsis, 17 in rice and 9 in maize. The mature microRNA is 21 nt long and is always located in the 5' fold back arm.

The *MIR169* gene family targets the nuclear factor *NF-YA* (*CBF-B* in mammals, *HAP2* in yeast) gene family of transciption factors (Jones-Rhoades and Bartel, 2004). It has been shown that MtmiR169 regulates the temporal and spatial expression pattern of a member of the NF-YA family during nodule development by a "clearing mechanism" (Combier et al., 2006).

NFYAs are general transcription factors that bind DNA as part of the trimeric NF-Y complex together with NF-YB (CBF-A, HAP3) and NF-YC (CBF-C, HAP5). NF-YB and NF-YC form a protein dimer to which NF-YAs show high interaction affinity (Mantovani, 1998; Mantovani, 1999) (Figure 1.7B). NF-YA contains a highly conserved domain at the C-terminus, which can be divided in two halves: one for the interaction with the NF-YB and NF-YC protein dimer and one for DNA binding. NF-YA and NF-YC contain loosely conserved glutamine rich domains (Q-rich domain) for transcriptional activation at their N-terminus and C-terminus, respectively. NF-YB and NF-YC contain the Histone Fold Motif (HFM) for protein interaction (Mantovani, 1998; Mantovani, 1999) (Figure 1.7A).

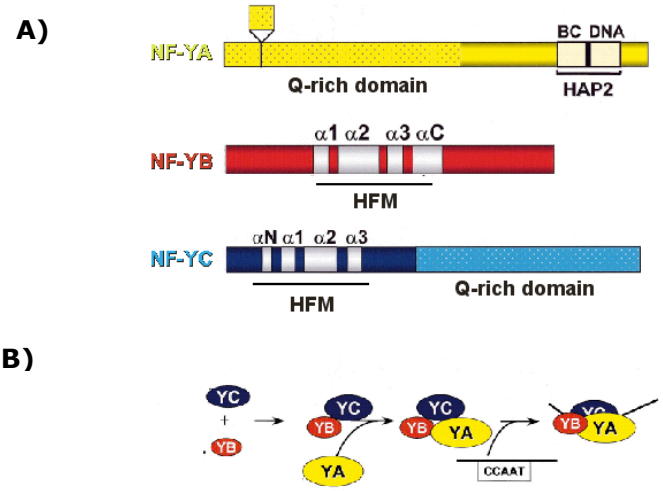


Figure 1.7: Schematic diagram. The NF-Y proteins are shown in the upper panel. The light yellow box of NF-YA indicate the conserved domain which allows interaction with the NF-YB/NF-YC dimer and DNA binding (see text). Grey boxes in NF-YB and NF-YC indicate the α–helices of the histone fold motif (HFM). The Q-rich domain in NF-YA and NF-YC is for transcriptional activation. The lower panel shows association of the NF-Ys subunits and binding to the DNA. From Mantovani, 1999.

Mammals and yeast contain only one gene copy of *NF-YA*, *NF-YB* and *NF-YC*, while Arabidopsis contains ten copies of *NF-YA*, ten of *NF-YB* and nine of *NF-YC* (Edwards et al., 1998; Gusmaroli et al., 2001; Gusmaroli et al., 2002). It is not known whether family members bind DNA in distinct combinations for specialized functions or whether they randomly associate. Several examples in plants indicate regulatory functions of NF-Y complexes. *LEAFY COTYLEDON1* (*LEC1*) is a critical regulator during early and late stages of embryo development and encodes a NF-YB subunit. Based on their protein interaction domains NF-YB family members can be separated in LEC1-type and non-LEC1-type and only those with the LEC-1 type domain are able to rescue the wild-type phenotype in a *lec1* background. Whether this is due to interaction with specific transcription factors or to the ability of the LEC1-type domain to bind specific DNA sequences is not known (Kwong et al., 2003; Lee et al., 2003). NF-YAs compete with CONSTANS (CO) (Putterill et al., 1995) in binding the NF-YB/NF-YC dimer to regulate flowering time. Yeast two hybrid screening did not reveal preferential combination of CO to specific NF-YB and NF-YC subunits (Ben-Naim et al., 2006; Wenkel et al., 2006).

The NF-Y complex binds to the CCAAT DNA motif which is present in at least 30% of eukaryotic promoters of developmentally and cell cycle regulated, housekeeping and inducible genes (Mantovani, 1998). A CCAAT direct repeat spaced ca. 30 basepairs (bp) from each other is also present in the second intron of *PLE*, *FAR*, *pMADS3*, *AG* and the class C-genes of other 29 Brassicaceae species (Hong et al., 2003). Experiments with the GUS reporter gene fused to the *AG* second intron showed that the CCAAT repeat is important for AG expression in late stages of flower development in Arabidopsis (Hong et al., 2003). The CCAAT repeat is located next to the *LEAFY*/*WUSCHEL* (*LFY*/*WUS*) binding site which is responsible for *AGAMOUS* activation in the small region of stem cells where the expression of these two genes overlap (Lohmann et al., 2001; Mayer et al., 1998; Weigel et al., 1992). Putative binding sites for FLORICAULA/ROSALUTA (FLO/ROA) (Coen et al., 1990; Kieffer et al., 2006), the Antirrhinum counterparts of LFY/WUS, are also located in the second large intron of *PLE* (Figure 3.4).

The presence of a CCAAT repeat suggests that the NF-Y complex is involved in the regulation of *PLE* and *FAR* activation and/or maintenance together with other transcription factors. *FIS* is supposed to regulate the expression levels of one of the components of the NF-Y complex and thereby to indirectly control *PLE* expression. Identification by map-based cloning and molecular characterization of

FIS and analysis of its function by genetic or molecular studies is described in the next chapters.

2. MATERIAL AND METHODS

2.1 Chemicals, enzymes, oligonucleotides, cloning vectors

Chemicals used for this work were purchased from Sigma-Aldrich (Steinheim), Merck (Darmstadt), Serva (Heidelberg), Duchefa (Haarlem, The Netherlands), Biozym (Hamburg), Roth (Karlsruhe), Eppendorf (Hamburg), FMC Bioproducts (Brussels, Belgium), Gibco BRL (Karlsruhe) and Invitrogen (Karlsruhe). Enzymes were purchased from Roche (Penzberg), Takara Bio Europe/Clontech (Saint-Germain-en-Laye_France), New England Biolabs (Frankfurt am Main), Fermentas (St. Leon Rot), Stratagene (Heidelberg), and Invitrogen (Karlsruhe). Oligonucleotides were synthesized at Metabion (Martinsried) and Invitrogen (Karlsruhe). Cloning vectors used were pGEM-T easy (Promega), pDONR-201 (Invitrogen) and pENSG-YFP (N.Medina-Escobar and J. Parker, unpublished).

2.2. Buffers, solution and media

Standard buffers, solutions and media were prepared as described by (Sambrook, 1989).

2.3 Plant Material

Antirrhinum majus plants were grown in the greenhouse at 18-25°C with additional light provided during winter. For the experiment described in 3.6.2 plants were grown in a climate chamber at 22°C during the day and 16°C during the night under a 16h light/8h dark regime. For observations with *ple*-625 (see below) plants were grown at 16°C (non-permissive temperature) or at 26°C (permissive temperature) under a 16h light/8h dark regime (without dropping the temperature in the dark) to prevent or to facilitate transposon excision, respectively.

The wild type line Sippe50, and the mutants *fis*-1 and *ple*-1 were obtained from the Gatersleben (GDR) seed collection. The progenitor of the wild type line 165E (JI98), *fim*-679 and ple-625 were kindly provided by Rosemary Carpenter (Norwich, UK).

fis-1 is a stable allele (Motte et al., 1998) and *fis*-2 is a newly isolated unstable allele (Z. Schwarz-Sommer, unpublished) with a reversion frequency of 1:500 (see 3.1.2).

Ple-237 (Lönnig and Saedler, 1994) carries a new CACTA-type transposon insertion in the second large intron of the *PLE* gene (Figure 3.5a) and *ple*-1 (Stubbe, 1966) is generated by a Tam2-like transposon insertion in the last intron of *PLENA* (Z. Schwarz-Sommer, unpublished).

Ple-625 (Bradley et al., 1993) carries a Tam3 insert in the second intron of the *PLE* gene, whose excision can be controlled by growth at different temperatures; at 16°C the insert is stable and at 26°C frequent excisions occur.

Fim-679 is due to a deletion in the *FIM* open reading frame (Simon et al., 1994).

To generate double mutants the mutant parents were crossed to obtain an F1 plant, which was subsequently self-fertilised. The resulting F2 population (100-200 plants) was inspected for the occurrence of plants with flower phenotypes distinguishable from the phenotype of the parental lines. The genotype of the putative double mutants was confirmed by backcrossing to parental lines or by genotyping by Polymerse Chain Reaction (PCR). In addition, to obtain large number of double mutants, six or more individuals homozygote for one parental mutation and heterozygote for the other were selected from the F2 population and selfed.

The following F1 hybrids were generated:

***fis*/+; *ple*/+** by crossing *fis* individuals with *ple*/+ pollen and genotyping the resulting plants for the presence of the *ple* mutant allele

***fis*/+ ; *fim*/+** by crossing *fim* plants with *fis* pollen

2.4 Scanning Electron Microscopy (SEM)

Fresh petal tissue from wild type and *fis*-2 mutants was gold coated in a sputtercoater (SCD 004, Balzers/Liechtenstein) and SEM was performed with a Zeiss DSM 940 apparatus.

2.5 Bacterial transformation

2.5.1 Preparation of electro-competent cells of *E.coli* and *A.tumefaciens*

Bacteria (Table 2.1) were grown to mid-log phase, chilled, centrifuged, and then washed extensively under sterile conditions to reduce the ionic strength of the cell suspension and to destabilize the cell wall. For this purpose, cells from a glycerol stock were inoculated in 300 ml of LB medium and cultured overnight. Next day 5-50 ml of the culture was used to inoculate 500 ml of LB media and grown at 16 °C until the OD_{600} reached 0.4. Freshly grown bacteria were pelleted and washed with 20 ml of distilled water, centrifuged down and the pellet resuspended in 1 ml of cold distilled water containing 7% DMSO. The cells were aliquoted in 50 μl in sterile tubes, quickly frozen in liquid nitrogen and stored at –70°C.

Table 2.1: Genotypes of bacterial strains

E.coli DH5α	SupE44 ΔlacU169 (80 laZΔM15) hsdR17 recA1 EndA1 gyrA96 thl-1 relA1.
E.coli DH10B	F-mcrA D (mrr-hsdRMS-mcrBC) F80diacZDM15 DlacX74 endA1 recA1 D (ara, leu) 7697araD139 galU galK nupG rpst T1R
A.tumefaciens	GV3101:pMP90-RK (Koncz et al., 1994)

2.5.2 Electroporation of bacterial cells

Frozen electro-competent cells were thawn on ice and mixed with 1 μl of ligation mix. The mixture was transferred into a prechilled cuvette. Electroporation was done at 1800V and 1 ml of LB medium was immediately added. After incubation at 37°C (or at 28°C for *Agrobacterium*) for 1 hour (or 2 hours for *Agrobacterium*), the cells were plated onto selective media.

2.6 DNA and RNA isolation

Plant genomic DNA was isolated using the DNeasy Plant Maxi kit (Qiagen). Plasmid DNA was isolated using the Mini/Midi Plasmid DNA purification kit (Macherey-Nagel, Düren) after over night culture in LB medium containing the appropriate

antibiotics for selection. PCR clean up and agarose gel extraction was performed with the NucleoSpin®ExtractII (Macherey-Nagel).

The quality and quantity of DNA were judged by comparison of band intensity on ethidium bromide (EtBr) stained agarose gel with a DNA molecular weight standard (Fermentas).

Total RNA from flowers and inflorescences was isolated using the RNeasy Plant Mini kit from Qiagen. polyA$^+$ RNA was obtained using magnetic beads (Dynabeads® Oligo(dT)$_{25}$, Invitrogen). Concentration and purity of the isolated RNA was determined by a standard spectrophotometric measurement (Sambrook, 1989).

2.7 Southern analysis

2.7.1 Digestion of genomic DNA and PCR products

Digestion of DNA with restriction endonucleases was carried out according to the manufacturer's instructions and in the provided buffers.

Digestion of genomic DNA (gDNA):	Digestion of PCR products:
1-2 μg DNA	5 μl of PCR product
5 μl 10X buffer	2.5 μl 10x buffer
10 Units enzyme	5 Units enzyme
add H_20 to 50 μl	add H_20 to 25 μl

2.7.2 Blotting and hybridization of the membrane

The DNA fragments were separated on an agarose gel and alkaline blotted to Hybond N$^+$ nylon membranes (Amersham). Alkakine transfer was performed as described in (Sambrook, 1989).

The blot was pre-hybridised in hybridisation buffer (Table 2.2) for 2 hours and then hybridised in the same buffer containing radioactively labelled probe overnight at 65°C. The membrane was rinsed twice in washing buffer and then washed twice at 65°C for 20 minutes. After washing the membrane was exposed to Kodak Biomax MS film with intensifying screens.

Stripping was performed at 80°C in a stripping buffer and utilised for a further hybridisation. The buffers were prepared as follows:

Table 2.2: Buffers used for hybridisation

Hybridisation buffer (1l):	Washing buffer (1l):	Stripping buffer (1l):
150 ml 20X SSPE	25 ml 20X SSPE	5 ml 20X SSPE
200 mg PVP 90	10 ml 10% SDS	10 ml 10% SDS
200 mg Ficoll 400	add H_20 to 1 liter	add H_20 to 1 liter
10 ml 10% SDS		
add H_20 to 1 liter		

2.7.3 Radioactive labelling of DNA probes

Probes were prepared from plasmid templates or from PCR products using the following protocol for random oligonucleotide-primed synthesis:

50 ng of DNA
add H_20 to 20 μl and boil for 5 minutes.
Chill quickly on ice and add the following mixture:
3 μl 10X oligo buffer
1.6 μl Klenow (2U/μl)
5 μl [α_{32} P]-dCTP (10 μCi/μl)

The Klenow enzyme was used for the fill-in reaction. After 1-2 hours at room temperature, the labelling product was purified using a NucleoSpin®ExtractII column.

2.7.4 Radioactive labeling of the DNA ladder

Labeling of the 1 kb DNA ladder was performed as follows:

3 μg 1 kb DNA ladder (Invitrogen)
5 μl 10X Klenow buffer
2 μl Klenow polymerase (2U/μl)
5 μl [α_{32} P]-dCTP (10 μCi/μl)
add H_20 to 50 μl

10X Klenow buffer:
0.5M Tris pH 7.5
0.1 $MgCl_2$

The reaction was performed at room temperature for 30 minutes. An aliquot was then analyzed at the scintillation counter and the reaction mixture diluted to 1000 cpm/μl with TE 0.1. To 2 μl of the DNA ladder, 7 μl of loading dye and 18 μl of TE 0.1 were added. The DNA ladder was then ready to be loaded on an agarose gel.

2.8 Standard PCR

Home made Taq Polymerse was used for amplification of small DNA fragments. Large DNA fragments were amplified with TaKaRa LA Taq™. Standard PCR reactions were performed in 25-50 μl volume, under the following conditions:

PCR mixture:	Cycling parameters:
5 μl 10X PCR buffer	95 °C 5 min
5 Units Taq DNA polymerase	95 °C 40 sec
1 μl 10 mM dNTP mix (dATP, dCTP, dGTP, dTTP)	Ta 30 sec (n)
0.5 μl 10 μM sense primer	72 °C 30-90 sec
0.5 μl 10 μM antisense primer	72°C 5 min
20-50 ng genomic DNA or 1-2 ng plasmid DNA	10 °C ∞
add H_20 to 50 μl	

Ta (annealing temperature of the primers) and "n"can vary for individual reactions.

2.9 Nucleic acid sequencing

DNA sequences were determined by the ADIS MPIZ DNA core facility on Applied Biosystems (Weiterstadt) Abi Prism 377, 3100 and 3730 sequencers using BigDye-terminator v3.1 chemistry.

2.10 Sequence analysis

Routine sequence analysis was performed using the MacVector™ 7.2.3 and Assemblylign™ 1.0.9c programs. Database searches were routinely carried out using the BLAST algorithm (Altschul, 1997) at GenBank

(http://www.ncbi.nlm.nih.gov) and GenAgent, the Antirrhinum EST database (http://charon.mpiz-koeln.mpg.de/GenAgent/index.php3).

2.11 Fine mapping

2.11.1 Amplified fragment length polymorphisms (AFLP) analysis

- DNA digestion and ligation of adaptors

250 ng of genomic DNA were incubated 1h at 37°C with 5 units of *EcoRI* (or 5 units of *PstI*) and 5 units of *MseI*, in 40 µl reaction volume.

Restriction reaction (40 µl):

4 µl 10X RL buffer → <u>10X RL buffer:</u>
0.5 µl *EcoRI* (or *PstI*) (10U/µl) — 100mM Tris-Hcl
0.5 µl *MseI* (10U/µl) — 100mM MgAc
250 ng/µl — 500mM KAc
add H_20 to 40 µl

10 µl of a solution containing 5 pMol of *EcoRI*-adaptors (or *PstI* adaptors), 50 pMol of *MseI*-adaptors, 1 µl of 10 mM ATP, 1 µl of 10X RL buffer, and 1 unit of T4 DNA ligase was added to the restriction solution and the incubation was continued for 3h at 37°C. Adaptors were prepared by mixing equal molar amounts of both strands, denatured at 95°C for 5 minutes and renatured at room temperature over 10 minutes.

Ligation reaction (50 µl):

40 µl restriction mixture
1 µl *EcoRI*-adaptors (5 pM/µl)
1 µl *MseI*-adaptors (50 pM/µl)
1 µl 10 mM ATP
1µl 10X RL buffer
1 unit of T4 DNA ligase
After ligation, the reaction mixture was diluted 10 fold with TE0.1 buffer and stored at –20°C.

- Pre-selective amplification

The cycling parameters were chosen as described in (Vos, 1988). The reaction mixture was performed in 20 μl as follows:

2 μl 10 X PCR buffer

0.4 ul 50 mM $MgCl_2$

0.8 μl 10 mM dNTPs

0.4 μl 10 μM each primer

0.4 μl Taq Polymerase (5U/μl)

4 μl template DNA

add H_20 to 20 μl

The amplification products were diluted 20-fold with TE0.1 buffer before proceedings to selective amplification.

- Labeling of primers and the size marker with [γ_{33} P]-ATP

The labeling reaction was performed in 12.5 μl. The mix was incubated at 37°C for 45 min and then stopped by incubation at 75°C for 10 min. The reaction mix was prepared as follows:

1.3 μl 10 X T4 PNK buffer

0.4 μl T4 polynucleotide kinase (PNK) (6 U/μl)

2.5 μl *EcoRI* (or *PstI*)-primer (10 pM/μl)

2 μl [γ_{33} P]-ATP (10 μCi/μl)

add H_20 to 12.5 μl

<u>10 X T4 PNK buffer:</u>

250 MM Tris-HCl Ph 7.5

100 mM $MgCl_2$

50 mM DTT

5 mM Spermidine-HCl

30-330 AFLP ladder, not dephosphorylated, was radioactively labeled by exchange reaction.

The reaction mix was prepared in a volume of 5 μl as follows:

1 μl 5 X Exchange reaction buffer

2 μl 30-330 AFLP DNA ladder

1 μl [γ_{33} P]-ATP (10 μCi/μl)

1 μl T4 polynucleotide kinase (6 U/μl)

<u>5 X Exchange reaction buffer:</u>

250 mM imidazole (pH 6.4)
60 mM $MgCl_2$

50 mM 2-mercaptoethanol

350 μM ADP

The reaction was incubated for 10 min at 37°C and then stopped by heating the tube at 65°C for 15 min. Before loading the reaction mixture was mixed with an equal volume of loading buffer (see below).

- ***Selective amplification***

Selective amplification was carried out with two oligonucleotide primers to which two selective nucleotides were added at their 3' end. One of the two primers (e.g. *EcoRI*-primer or *PstI*-primer) was end-labeled. The cycling parameters were as described in (Vos, 1988). PCR was performed in 20 μl of reaction volume:

2 μl 10 X PCR buffer
0.4 μl $MgCl_2$
0.4 μl 10 mM dNTPs
0.6 μl 50 ng/μl not-labeled primer
0.5 μl labeled primer
0.2 μl Taq polymerase (5 U/μl)
5 μl diluted amplification products
add H_20 to 20 μl

Primer names and sequences are listed in the Appendix 6.2.

- Denaturing polyacrylamide gel analysis of AFLP fragments

AFLP selective amplification products were mixed with an equal volume (20 μl) of formamide loading buffer. The samples were denatured for 5 min at 95°C and then quickly chilled at 4°C on ice. 4 μl of each sample were loaded on a 5 % denaturing polyacrylamide gel.

Loading buffer:	AFLP gel mix:	5%acrylamide/bis (20:1):
98% formamide	75 ml 5% acrylamide/bis	250 ml acrylamide/bis
10 mM EDTA pH 8.0	50 μl TEMED	450 g urea
0.025% bromophenol blue	750 μl 10% APS	50 ml 10 X TBE for AFLP
0.025% xylene cyanol		add distilled water to 1 liter, filter sterilize and store at 4°C.

Gels (31cm x 38.5cm x 0.4mm) were fixed onto one of the two glass plates, one of which had been treated with sticking solution (0.3% acetic acid and 0.015% methacryloxypropylmethoxysilane) whereas the other one was treated with repellent solution (Acrylease ^{TM}, Stratagene). The gel electrophoresis apparatus was provided by BioRad (Munich). A pre-run was performed for 30 min in 0.5 X TBE buffer before loading the gel. The gel was then run at constant power (58 W for 3-4 hours). After electrophoresis, the gel on the glass plate was fixed for 30 min in 10% acetic acid and then rinsed in water. After drying over-night at 80°C, glass plates were exposed for 1-2 days to Hyperfilm-MP (Amersham Bioscience, Munich) before developing. Glogos II Autorad markers (Stratagene) were applied on the glass plate before exposure for autoradiography in order to allow a precise alignment between the film and the gel.

2.11.2 Isolation of AFLP fragments

- Extraction and cloning of AFLP fragments from polyacrylamide gel

AFLP fragments were excised directly from the polyacrylamide gel on the glass plate using a razor blade, placed in a 1.5 ml eppendorf tube with 250 µl TE 0.1 buffer and boiled for 10 min. After spinning, the supernatant was transferred to a new tube, precipitated in 0.7 volume isopropanol + 1/10 volume NaAC 3 M (pH 5.2) and stored for 2 hours at –20 °C. The fragment was pelleted by centrifugation at 13000 rpm for 30 min and then resuspended in 20 µl TE0.1.

1 µl was used for amplification. Reaction conditions and the cycling parameters were as described in 2.11.1 for selective amplification except that the oligonucleotide primers were not end-labeled. After electrophoretic separation and extraction from a 1.2% agarose gel, the PCR products were cloned into pGEM-T easy Vector and transformed into *E.coli*.

- E.coli colony screening and colony hybridization

Single white colonies were picked from the plate, placed in a 96-well microtiter plate (Nunc, Wiesbaden) filled with selective LB medium and incubated at 37 °C for 2-3 hours. 1 µl of the liquid culture was then pipeted into a PCR mixture containing the universal T7 and SP6 primers. PCR products were tested on

agarose gel. 5 μl of the PCR products were then spotted on a Hybond-N^+ nylon membrane (Amersham) and cross-linked in the UV Stratalinker 2400 (Stratagene) The selective reaction products (amplified without end-labeling of the primers) of the parental lines (e.g. 165e and *fis*-1) were also included as control.

- Preparation of the probe

For plasmid DNA extraction single colonies were inoculated over night in 5 ml LB medium containing the appropriate antibiotics. Plasmid DNA was restricted with *NotI* and *SacI* (2.7.1) and the restriction products were electrophoretically separated on a 1.2% agarose gel: after gel extraction (2.6), the insert was radioactively labeled with [α-32P]-dATP. (2.7.3)

Hybridization conditions were as described in 2.7.2.

A schematic example of detection of a clone corresponding to the marker of interest is shown below:

1 ●	2	3 ●	4	5
6	7	8 ●	9	10 ●
11	12	13	165e ●	*fis*-1

A clone (e.g. #1) corresponding to the marker of interest, will hybridize to itself and to some of the other tested clones (#3,#8 and #10) and as well as to the 165E control PCR, but not to the *fis*-1 control PCR.

2.11.3 Identification of Cleaved Amplified Polymorphic Sequences (CAPS marker)

PCR products were digested with restriction endonucleases as indicated in 2.7.1. for at least 3 hours at the recommended temperature and analyzed after electrophoresis on agarose gels. Recombinants and parental lines were then tested for differences in their restriction pattern.

2.12 Chromosome walking

2.12.1 Bacterial Artificial Chromosome (BAC) library screening

An Antirrhinum wild type genomic *HindIII*-BAC library cloned in pIndigoBAC was previously spotted onto nylon membranes (kindly provided by Dr. R. Castillo).

Labeling of the probes was performed as indicated in 2.7.3 and hybridization, washing and stripping of the BAC filters was performed as described in 2.7.2.

Filters were exposed to a Storm screen (Amersham) and analyzed with the Typhon Phosphoimager Scanner (Molecular Dynamics).

Standard PCR analysis of the identified BAC plasmids with the same primer pair used to synthesize the probe confirmed positive BAC clones (Appendix 6.3 and 6.4).

Plasmids of positive BAC clones were isolated and insert ends were sequenced with the flanking universal primers T7 and T3 (Appendix 6.1).

To define the relation of the positive BAC clones to the *FIS* contig, the clones were restricted with *HindIII*, southern blotted and hybridized (2.7) with the same probe used to screen the BAC library.

In the Appendix 6.4 are listed the isolated BAC clones, their size and the probes used for their detection.

2.12.2 Expressed Sequence Tag (EST) library screening

A filter-arrayed Antirrhinum EST library representing ~12 000 unigenes expressed in 12 different vegetative and reproductive organs from defined developmental stages was screened (Bey et al., 2004).

Labeling of the probe was performed as indicated in 2.7.3. Before labeling the BAC plasmids were heated for 5 minutes at 90°C in order to introduce nicks in the plasmid.

Hybridization, washing and stripping of the EST filters was performed as described in 2.7.2. EST sequences were analyzed in GenAgent, primers were derived and

confirmation of the positive clones was performed by PCR using the BAC clone as template. Appendix 6.5 lists the ESTs hybridizing to the tested BAC clones.

2.13 Genome Walker

Final isolation of *FIS* was performed using the Genome Walker™ kit (Clontech) according to the manufacturer's instructions. Basically, 6 adapter-ligated libraries were prepared after restriction with the following enzymes: DraI, EcoRV, PvuII, StuI, HincII, NaeI. These libraries, provided by Dr. M. Vandenbusche (Nijmegen, the Netherlands), were used for PCR amplification with the Genome Walker adaptor primer (AP1) and the Gene Specific primer (MIBUS474, Appendix 6.6). In these reactions Advantage® Genomic LA Polymerase was used for amplification.

The touchdown profile is shown below:

Reaction mixture (29 μl volume):

3 μl 10X buffer

0.6 μl 10 mM dNTP mix

0.6 μl GSP MIBUS474

0.6 μl KlenTaq poymerase

add water to 29 μl.

PCR profile:

7x (94°C 2 sec, 72°C 3 min)
5x (94°C 2 sec, 72°C-1°C/cycle 3min)

30x (94°C 2 sec, 67°C 3 min)

6x (94°C 2 sec, 66°C 3 min)

10 ∝

2.14 cDNA phage library screening

An EMBL3 phage library containing genomic Antirrhinum cDNA fragments (Dr. H. Sommer) was screened according to (Sambrook, 1989). PCR fragments representing the following seven *A. thaliana* NF-YAs were prepared by Reverse Transcribed (RT)-PCR and used as probe for heterologous screening (2.7.3):
At1g17590 (YA1F/R); At1g72830 (YA2F/R); At3g14020 (YA3F/R); At1g54160 (YA4F/R); At2g34720 (YA5F/R); At1g30500 (YA6F/R); At3g05690 (YA7F/R). The forward (F) and reverse (R) primers are listed in Appendix 6.6.
Phage propagation was in the *E.coli* strain POP13. Several dilutions of the phage library were used to titer the library to obtain approximately 200 000 plaques/20 plates. Three rounds of screening were necessary to obtain single positive plaques. Single plaque lysates were amplified with primers 233/234 (derived from

NM1149 phage sequences flanking the cDNA insert) and sequenced with primer AD22c (the 5′ adaptors used to prepare the cDNA mix for cloning).
Partial, 5′ end truncated sequences of the NF-YA1, NF-YA3 and NF-YA4 were already available in the Antirrhinum EST library. 3′ end sequencing of the phage clones and when necessary 5′ RACE were performed to get the sequence of the full-size transcripts (Appendix 6.6).

2.15 5′ Rapid Amplification of cDNA Ends (5´RACE)

To determine the 5′ end of the cDNA ends the SMART procedure (Clontech) was applied on poly(A)$^+$ RNA; PCR amplification was then performed with the SMART 5' oligonucleotide as follows:

PCR mixture (50 μl volume):

5 μl	diluted 1st strand
5 μl	10X buffer
1 μl	10 mM dNTP mix
1 μl	10 μM SMART primer
1 μl	10 μM antisense primer
1 μl	KlenTaq polymerase
36 μl	H_20

Cycling parameters:

95°C 1 min
95°C 20 sec
65°C 30 sec
68°C 4:30 min
10 °C ∞

n

The resulting fragments were subcloned into the pGEMT vector (Promega) and transformed into *E.coli*.

2.16 Expression analysis

For quantitative and semi-quantitative analysis 30-50 young inflorescences, 3-5 mm in length, grown in the field were collected during the summer season. Samples were pooled in order to minimize the biological component of variance. For each pool (e.g. genotype) three technical replicates were performed. Stage 2 flower buds (Bey et al., 2004) were collected from plants grown in the greenhouse and buds for organs dissection from plants grown in a climate chamber.

2.16.1 cDNA single strand synthesis

cDNA was prepared from total RNA. cDNA was synthesized using SuperscriptII (Invitrogen). The reaction was performed as follows:

1 μg total RNA

1 μl 10 μM primer oligo$(dT)_{20}$

add RNase/DNase free water to 10 μl.

Incubate at 70°C for 10 minutes and directly place on ice for 2 minutes.

Add the following to the tube on ice:

4 μl of 5X First Strand buffer

2 μl 20 mMDTT

2 μl 10 mM dNTP mix

1 μl SuperscriptII

Incubate for 1 hour at 42°C. After precipitation in EtOH, dissolve in 300 μl TE 0.1 and store at –20°C.

2.16.2 Semi-quantitative RT-PCR (SQ-PCR)

SQ-PCR was performed in 50 μl with the Advantage® cDNA PCR Kit & Polymerase Mix for amplification of cDNA templates.

PCR mixture (50 μl volume):

10 μl	diluted 1st strand
5 μl	10X buffer
1 μl	10 mM dNTP mix
1 μl	10 μM sense primer
1 μl	10 μM antisense primer
1 μl	KlenTaq polymerase
31 μl	H_20

Cycling parameters:

95°C 2 min

95°C 30 sec

Ta 40 sec

72°C 30sec

n

10 °C ∞

RAN3 (Appendix 6.6) was used as control (e.g. housekeeping gene) for 1st strand cDNA concentration. Cycle numbers varied according to the expression level of the gene under analysis.

5 μl of the PCR products were loaded on 1,2 % agarose gel. In order to quantify band intensity, gels were scanned with a Phosphoimager Scanner (Molecular

Dynamics) and gel pictures were analyzed with ImageQuant (Molecular Dynamics).

2.16.3 Quantitative RT-PCR (qRTPCR)

qRTPCR was performed using iQ™ SYBR Green Supermix® and the BioRad Thermal Cycler. Expression data were analyzed using the iQ™5 Optical System. The reaction conditions were performed as indicated by the manufacturer.

Efficiency determination of the primer pairs and qRTPCR were performed using the same cycling parameters:

95°C 3 min

95°C 30 sec

58°C 30 sec

72°C 30 sec

Plate read. Repeat for 45 cycles.

Melt curve analysis: 55°C to 95°C, 0.5 °C/read.

The iQ5 software using the Pfaffi method performed relative quantification normalized to actin and GDPH. Appendix 6.6 lists the primers used in this analysis and their efficiency values (E).

2.17 *Agrobacterium* infiltration of *N.benthamiana* leaves

The constructs 35S::YFP-YA2wt and 35S::YFP-YA2mt were prepared using the Gateway cloning technology (Invitrogen), according to the manufacturer's instructions. Briefly, primer pair GW1/GW2 and GW3/GW2 were used to amplify wild type genomic DNA, the PCR products were purified with NucleoSpin®ExtractII columns and cloned into pDONR-201.

GW1/GW2 amplifies 91 bp of the NF-YA2 3' Untranslated Region (UTR) containing the MRE; GW3/GW2 amplifies the same template: GW3 contains 6 nucleotides at the 3' that include a mutation in the MRE (see Appendix 6.6).

35S::miRFIS construct was kindly provided by Dr. R. Castillo.

The constructs were transformed into the *A.tumefaciens* strain GV3101 (pMP90RK).

For the co-infiltration experiment, equal volumes of *Agrobacterium* cultures containing 35S::YFP-YA2wt (or 35S::YFP-YA2mt) (OD_{600}:0.03) and 35S::miRFIS (OD_{600}:0.26) were mixed before infiltration into *N.benthamiana* leaves as described by (Llave et al., 2002). Fluorescence was observed 2 days after infiltration using a Zeiss Axiophot microscope (filter EX 500/20, Beam Splitter 515 LP, EM 535/30).

3. RESULTS

3.1 Genetic studies : *FIS* as a repressor of class C- genes

3.1.1 The wild type Antirrhinum flower

Wild-type Antirrhinum flowers have been previously described in detail (Schwarz-Sommer et al., 1992). Briefly, the perianth consists of two abaxial, two lateral and one adaxial sepals in the first whorl (the calyx) and five petals in the second whorl with two adaxial and three abaxial lobes (corolla). The third whorl is made of four stamens and the staminodium. Two fused carpels form the gynoecium in the centre of the flower (Figure 3.1).

Figure 3.1: The wild type flower of *Antirrhinum majus*. (A) front view (B) lower petals removed to show the inner whorls. Floral whorls are indicated by numbers: sepals (1); petals (2); stamens (3); carpel (4).From Davies et al., 2006.

3.1.2 The *fistulata* mutant

For genetic and molecular studies we used the "classical" *fis*-1 mutant (McSteen et al., 1998; Motte et al., 1998; Stubbe, 1966) and the newly obtained *fis*-2 mutant (described in 2.3). In a field experiment with 10000 selfed *fis*-2 progeny plants, 20 wild type plants appeared, suggesting that *fis*-2 is a transposon-induced, genetically unstable allele. In contrast, *fis*-1 is a stable allele.

The first whorl of *fis*-1 and *fis*-2 flowers is composed of five sepals occasionally revealing stigmatic-like tissue at their tip. In the second whorl, anther-like structures develop at the tip of the ventral petals and petal lobe development is reduced. No homeotic defects are visible in the inner whorls (Figure 3.2). Stamenoid development in the second whorl indicates that in *fis* mutants class C-gene expression expands in the outer whorl, suggesting a function of *FIS* as a negative regulator of the C-function in the perianth of wild-type flowers (see introduction).

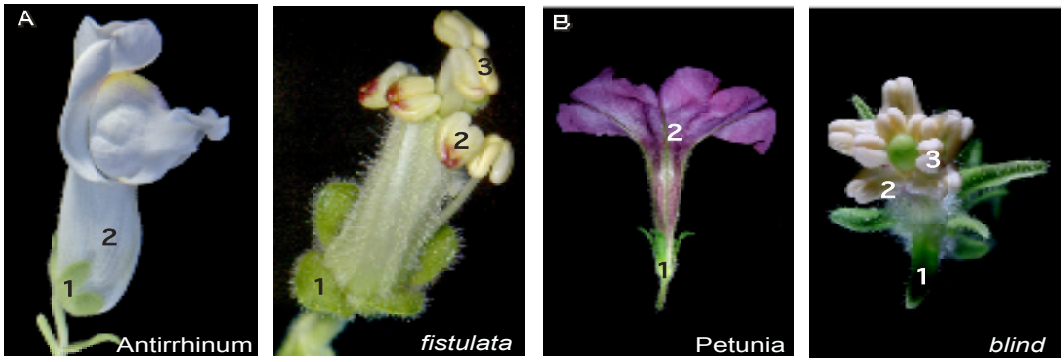


Figure 3.2: Phenotypic similarity of the Antirrhinum *fis* and the Petunia *bl* mutant. Notice anther-like structures in the second whorl. Genotypes are indicated and whorl numbered.

The phenotype of *fis*-1 and *fis*-2 mutant flowers is variable, sometime to the extent that is difficult to distinguish between wild-type and mutant plants. In *fis*-2 flowers pink stripes are sometime visible on the dark red petal lobes. Analysis by the SEM showed that the typical conical shape of petal epidermal cells is altered to flat and irregularly shaped cells within the stripes (Figure 3.3).

Figure 3.3: Phenotypic variability of the *fis*-2 mutant. A flower with the strong phenotype is shown at the left. The *fis*-2 mutant at the middle appears almost wild type-like, except for the slight deformation of upper and lower petals and the presence of light-colored stripes. SEM analysis of petal epidermal cells shown in the photograph at the right reveals typical conical petal cells adjacent to flat cells in the stripes.

However, comparison of the structure of these cells with epidermal cells of other floral organs did not reveal striking similarities. Whether these stripes are related to the homeotic conversion observed in the petals (and therefore to an impaired control of the C- genes) or to another biological function of *FIS,* is not clear.

3.1.3 The *plena* and *farinelli* mutants

Three *ple* alleles with transposon insertions were used for genetic analysis (Figure 3.4A, also see 1.1.2 and 2.3).

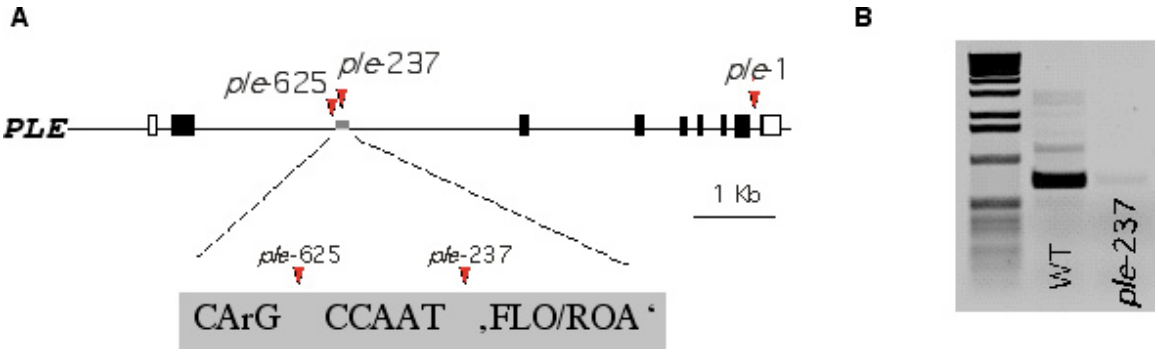


Figure 3.4: A) Schematic diagram of the *PLE* gene. Boxes represent exons and protein coding regions are shown in black. Red arrowheads indicate the position of transposon inserts in the mutant alleles. The small shaded box in the second intron shows the position of conserved putative cis-acting motifs highlighted in the grey box. **B) Expression of the PLE gene in wild type and *ple*-237 mutant inflorescences.** Qualitative RT-PCR was performed on 0.5 cm long young wild type (wt) and *ple*-237 inflorescences. Notice low level of wild type size PLE transcript in *ple*-237.

In *ple*-1 flowers the homeotic conversion of stamens to petals is not complete and anther-like structures producing fertile pollen develop (Figure 3.5A). The mutant allele contains a Tam2-like transposon insert suggesting that pollen production can be due to somatic reversion events. However, when *ple*-1 pollen was crossed to *ple*-1/*PLE* heterozygote, 50% mutants and 50% wild-tipe flowers appeared in the progeny suggesting that *ple*-1 is a genetically stable but leaky allele. In fact, we found very low expression of wild-type PLE transcripts by RT-PCR (not shown), which is possibly due to occasional transcriptional read-through and subsequent loss of the transposon along with the last intron during splicing.

In *ple*-237 and *ple*-625 flowers the homeotic conversion of the third whorl to petaloid organs is more complete (Figure 3.4C,E). Both alleles carry a transposon insert in the second *PLE* intron and are unstable, however, *ple*-237 reversion

events are extremely rare and *ple*-625 is stable when grown at 26°C. Semi-quantitative RT-PCR on *ple*-237 RNA samples indicates low level of wild-type PLE expression as compared to wild type (Figure 3.5B). Whether this is due to transposon excision or to splicing is not known, because *ple*-237 does not produce pollen.

Mutants of the *FARINELLI* gene (Davies et al., 1999) do not show any homeotic defects; when grown at higher temperature, pollen development is impaired and the flower is male sterile. The phenotype of *far* mutants suggests thus that PLE is fully functional in the absence of FAR. Therefore *FAR* has not been considered in this study.

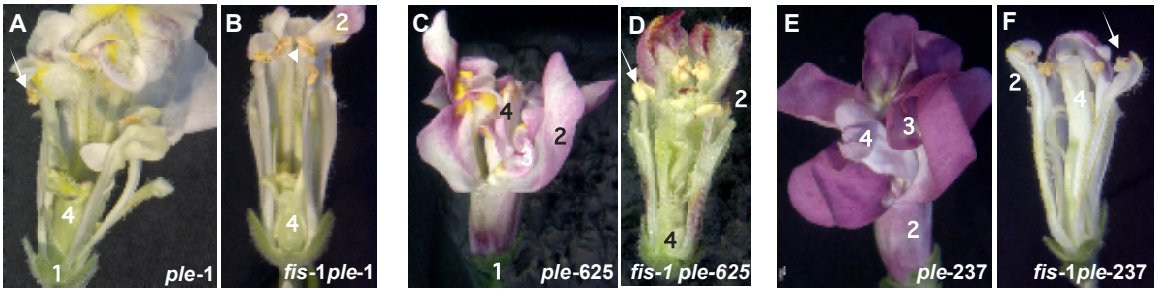
3.1.4 Genetic interactions between *FIS* and *PLE*

If homeotic alterations in the *fis* mutant are due to the lack of negative regulation of the C-function in the outer whorls, then homeotic defects should disappear in the C- mutant background. Previous observations with *fis ple* double mutants indeed indicated the lack of stamen to petal conversions (McSteen et al., 1998; Motte et al., 1998).

Our observations contrasts these findings: in all allelic combinations of *fis ple* double mutants stamen identity in the third whorl was rescued, and to some degree carpels formed in the fourth whorl. The second whorl occasionally revealed weak homeotic defects (Figure 3.5B, D, F). The flower was still indeterminate, however, suggesting that the threshold C-function necessary for termination of organ initiation in the centre of the flower was not reached.

To exclude the possibility that enhanced transposon excision events contributed to enhanced *PLE* activity we tested the genotype of the pollen produced by *ple*-625 *fis*-1 and *ple*-1 *fis*-1 double mutants by crosses to *ple* heterozygote plants; in both instances the *ple* mutant genotype of the double mutant pollen was confirmed.

These results suggest therefore, that in the published reports double mutants with partly rescued floral phenotypes were erroneously scored as *PLE* revertants. Furthermore, qRTPCR analysis (2.16.3) on *fis ple* RNA samples showed a dramatic increase of PLE expression as compared to *ple* (Figure 3.5G) suggesting that in the absence of *FIS,* PLE transcription (and thereby the chance for fortuitous loss of the transposon insert by splicing) is enhanced.



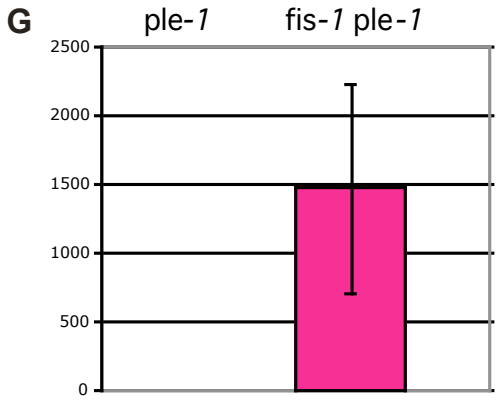


Figure 3.5: The effect of *fis* on C-gene expression in *ple* mutants. (A-F) Restoration of stamen identity in the third whorl of *fis ple* double mutants. The *ple* mutants are shown at the left and their respective double mutants with *fis*-1 at the right. Arrows indicate stamenoidy. Whorls are numbered. **(G)** PLE expression detected by qRTPCR in *ple*-1 and *fis*-2 *ple*-1 inflorescences. The error bar indicates standard deviation of technical replicates; the strong variation is due to the extremely low expression levels preventing reproducible amplification. The y-axis indicate the expression fold change in the double mutant as compared to *ple*-1.

These findings confirm *FIS* as negative regulator of PLE. Importantly, they also suggest that FIS, unexpectedly, negatively controls PLE in the inner whorls, in spite of the fact that mutation in *fis* does not affect the reproductive organs.

3.1.5 The *fimbriata* mutant

Fim-679 is a null allele and shows delayed expression of class B- and C- organ identity genes (Ingram et al., 1997; Simon et al., 1994). *Fim* flowers have a first whorl of sepals, and display numerous sepal-like internal organs in a spiral arrangement (Figure 3.6A, D). Late in development (e.g. in the upper flowers of the inflorescence) patches of petal tissue appear along with a reduced degree of indeterminacy with carpel-like structures occupying the centre of the flower.

It has been suggested that FIM, an F-box-containing protein, is involved in targeting repressors of B- and C- gene products for early proteolytic degradation (Ingram et al., 1997); in the absence of FIM, repressors of B- and C- are maintained, preventing the manifestation of the organ identity functions.

3.1.6 Genetic interaction between *FIM* and *FIS*

The *fim fis* double mutant was generated to test epistasis of *fis* to *fim* expecting, that by removing FIS as potential repressor of the C-function, reproductive organ identity will be restored in the *fim* background.

Interestingly, two novel phenotypes appeared in addition to the parental *fim* and *fis* phenotypes in the segregating F2 population. By genetic crosses we could ascribe these to double mutants (*fim fis*) and to plants that were *fim* mutant and carried *FIS* as heterozygote (*fim fis*/+). The outermost whorl of the double mutant contained normal sepals, whilst five carpels developed in the second whorl, which were fused to each other as well as to the third whorl carpels. No further organs initiated inside the third whorl. *Fim* mutants with one dosage of *FIS* resembled b-function mutants, such as *def* described in 1.1.2, bearing five sepals in the first and second whorl and initiating a third whorl of five carpels (Figure 3.6). The whorled organization of the flower was restored in both cases, suggesting that impaired C-function in the *fim* mutant is responsible for indeterminacy.

Carpel development in the *fim* background corroborates the wild-type function of FIS as a negative regulator of the C- function. As already observed by restoration of stamens in the *fis ple* double mutant (3.1.4), establishment of carpel identity in the third whorl of both the *def*-like (*fim*/- *fis*/+) and the double mutant (*fim fis*) flowers suggests a role of *FIS* in the inner whorls of the flower. In addition, *FIS* dosage dependence suggests that a threshold of C-expression level in the inner whorls is necessary to establish ectopic carpels in the second whorl.

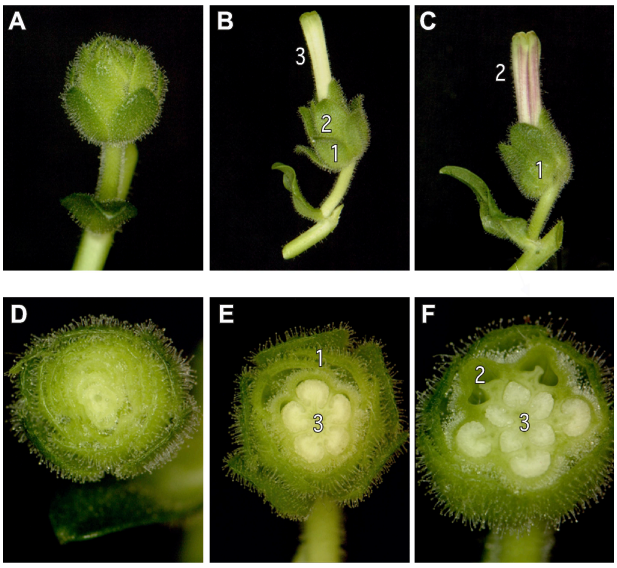


Figure 3.6: Rescue of organ identity defects of the *fim* mutant in the absence of the FIS function. In the *fim* mutant (A and D), B- and C organ identity function is impaired. The flower displays internal sepals in a spiral organization. Reduction of the *FIS* dosage (B and E) and its complete absence (C and F) results in different degrees of ectopic carpel development. D, E and F are cross-sections to reveal the inner organs.

3.1.7 Conclusions

Genetic and morphological analysis highlighted the following interesting features of the *FIS* control:

- The FIS function is necessary to prevent ectopic C-function in the outer whorls.
- Double mutant analysis (e.g. *fis ple*; *fim fis*) point to a role of *FISTULATA* in the centre of the flower, which was not clear in the *fis* single mutant due to the lack of homeotic defects other than in the second whorl.
- The dosage effect of *FIS* in *fim* plants suggests that dampening the C-expression level in the centre of the flower is important to maintain the C-expression boundary between the outer and the inner whorls in the wild type flower.

3.2 Molecular cloning of *FISTULATA*

Cloning of the *FISTULATA* gene was initiated with a map-based chromosome walking approach. In the following section a detailed description of the strategy to map *FIS* within Linkage Group (Lg) 6 of the *A. majus* genetic map is described followed by chromosome walking to a deletion of ~ 200 kilobases (kb) in the *fis*-1 allele. This effort represents the first reported attempt of this type in Antirrhinum.

3.2.1 The principles of map-based cloning

The map-based cloning strategy required as the first step the identification of molecular marker(s) that are tightly linked to *FIS*, the gene of interest. Subsequent steps then identify large genomic fragments and their relation to each other as well as to the *FIS* locus. The flowchart in Figure 3.7 highlights the approach we used routinely to proceed with chromosome walking toward *FIS*.

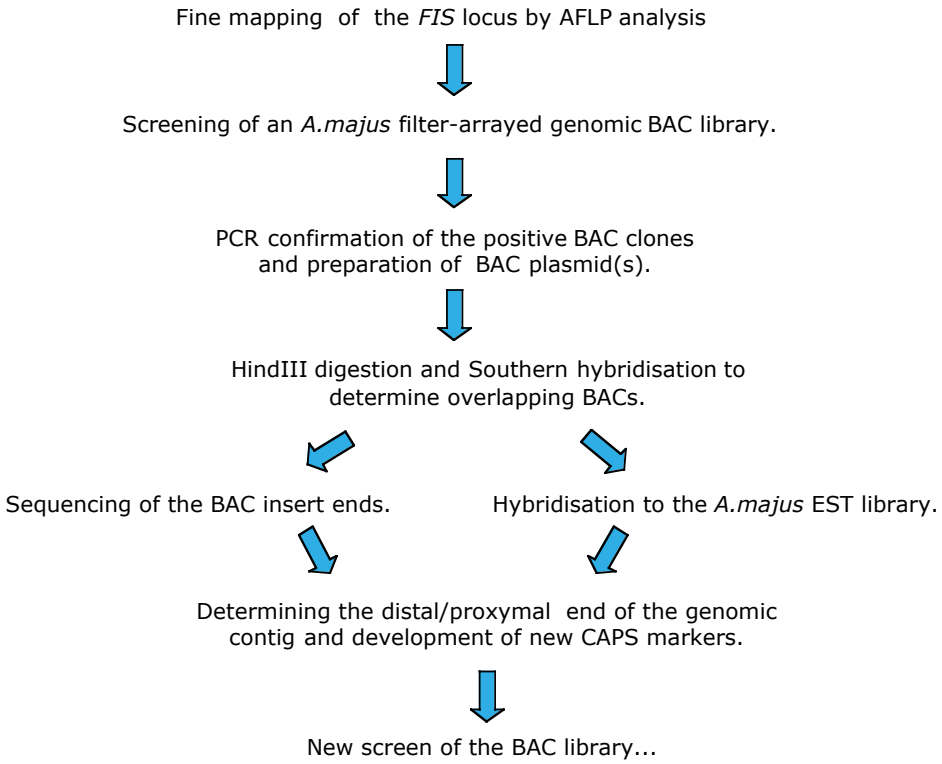


Figure 3.7: Flowchart indicating routine steps in chromosome walking.

3.2.2 Fine mapping of the *FIS* locus

Bulked segregant analysis of an F2 population segregating for *fis* generated two markers (CYC and 4089) mapping in the vicinity of the *FIS* locus (Dr. R. Castillo, personal communication). The CYC marker was previously found to map on Lg 6 during linkage analysis with an interspecific cross between *A. majus* x *A. molle* where 243 markers were ordered into 8 linkage groups (Schwarz-Sommer et al., 2003). Based on this linkage we could map *FIS* to Lg 6. For the identification of new markers we applied AFLP analysis because of its reproducibility and the high number of polymorphisms that can be detected.

Fine mapping of the *FIS* locus was performed after establishing a large F2 population by selfing a 165E x *fis*-1 F1 hybrid and selecting ~1700 *fis* mutants which were further used to map CYC and 4089 more precisely and to obtain recombinants for further fine mapping. According to this analysis CYC is located at 1.65 cM North from the *FIS* locus and 4089 maps at 0.41 cM South (Figure 3.8). The North/South orientation was arbitrarily established in order to distinguish between the two possible recombination regions (e.g. above and below *FISTULATA*).

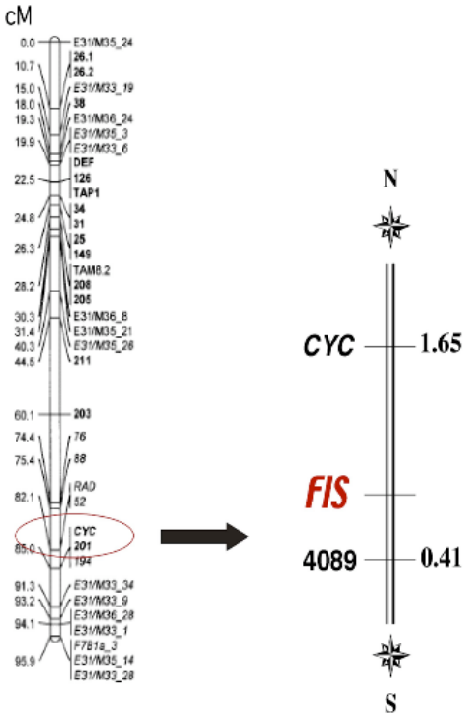


Figure 3.8: Fine map of the *FIS* locus. The previously established genetic linkage map of Lg6 (Schwarz-Sommer et al., 2003) is shown at the left with the region containing *FIS* encircled in red. The relation of the newly generated closely linked marker *4089* to *FIS* and *CYC* is shown at ther right.

AFLP screening of *fis* mutants heterozygote for CYC and 4089 (28 and 7 individuals, respectively) was then carried on to detect molecular markers which rarely recombined with *FIS* (1-10 recombinants in 1000 mutants is 0.1-1 cM). For AFLP analysis both *EcoRI* and *PstI* were used as hexa-cutter enzymes and *MseI* as the tetra-cutter for a total of 171 primer pair combinations (Appendix 6.2).

Two new AFLP markers were found in the vicinity of the *FIS* locus, named "marker e33" (AFLP primer pair *EcoRI*-33/*MseI*-31) and "marker p16" (AFLP primer pair *PstI*-16/*MseI*-31). According to the AFLP analysis "marker e33" maps 0.23 cM South (three recombinants) and "marker p16" 0.7 cM North (13 recombinants) of *FIS*. These AFLP markers were isolated and converted to "PCR friendly" CAPS markers to confirm their linkage to *FIS*. For this purpose the isolated AFLP fragments were subcloned, sequenced and served to derive primers for amplification on the parental lines (e.g. 165E and *fis*-1) to detect polymorphic sites.

- "marker p16"

"marker p16" hybridized more than once to the parental genomic DNAs when tested by southern blot experiments suggesting that this sequence is present in multiple copies in the genome (not shown). Accordingly, BAC library screening

yielded several BAC clones related to different genomic regions and it was not possible to define a contig linked to *FIS*. Physical mapping and chromosome walking from the North side of the *FIS* locus was therefore not accomplished.

- "marker e33"

Based on the nucleotide sequence of "marker e33", primer pair fw001/rev001 was designed and used for amplification and sequencing of the 165E and *fis*-1 parental fragments. Unfortunately, no polymorphism suitable as CAPS was found. Southern hybridization to the genomic DNA indicated that this marker corresponds to a single copy region (not shown). We therefore used "marker e33" to isolate a BAC contig that could be physically mapped in the proximity of *FIS*.

3.2.3 Physical mapping of "marker e33"

"Marker e33" was used to screen the BAC library and yielded one positive BAC clone (53b14). The clones were named after their coordinates in the library. Both ends of the 100 kb long BAC insert (Appendix 6.1 and 6.3) were sequenced and primers 004fw3/004rev3 and 005fw3/005rev3 were derived. No polymorphism was found within these sequences when comparing them in the parental 165E and *fis*-1 genomes.

To obtain additional, preferably single copy gene sequences contained in the 53b14 clone the BAC DNA was used to screen the *A. majus* ESTs library (Appendix 6.5). Primer pair 996/997 (Appendix 6.3), based on the sequence of EST 018_3_06_o16 amplified a 2 kb PCR product on BAC 53b14, confirming the relation of the EST to the BAC clone. A polymorphic site for *EcoRI* was found when comparing 165E and *fis*-1 nucleotide sequences of the 996/997 PCR product. Three out of the seven individuals heterozygous for marker 4089 were recombinants for *fis*. Thus physical mapping of marker 996/997 confirmed a genetic distance between the *FIS* locus and "marker e33" of 0.23 cM (Figure 3.9 and Figure 3.10).

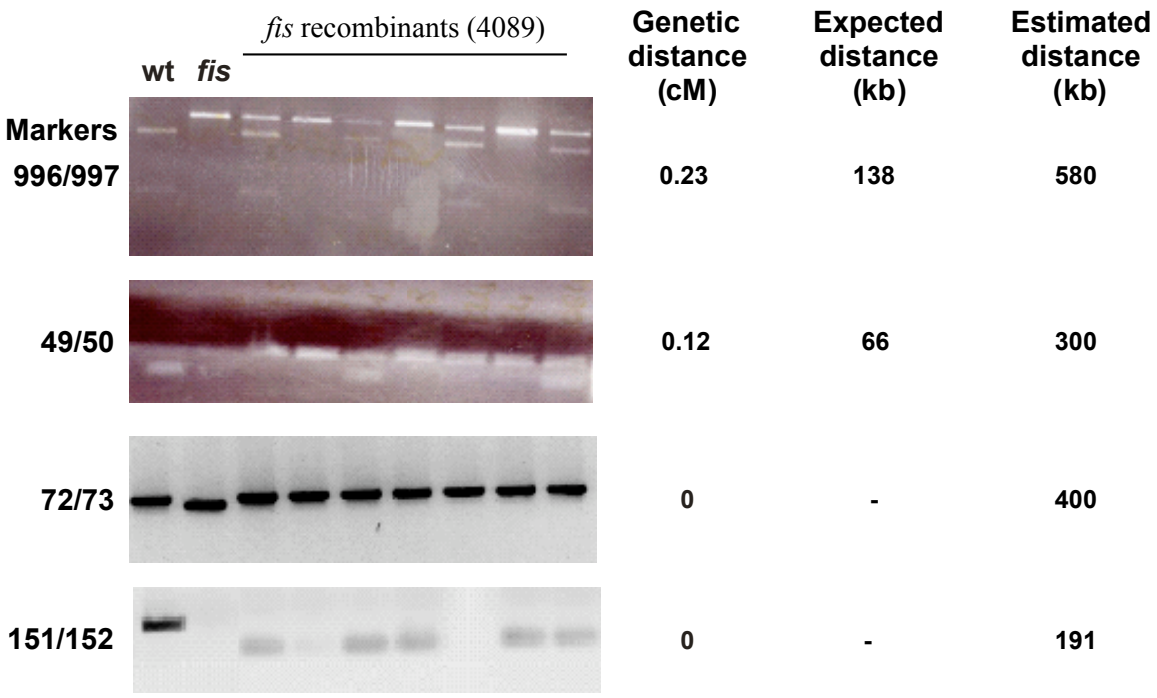


Figure 3.9: Markers used for chromosome walking toward the *FIS* locus. PCR reactions were performed on genomic DNA from the two parental lines and on the seven *fis* lines recombinant for the 4089 marker. Genetic distances to *FIS*, as well as the expected (theoretical) and the experimentally determined physical distances are indicated at the right for each marker. The resulting BAC contig is shown in Figure 3.10.

3.2.4 Chromosome walking

The genome size of *A.majus* is $3.6x10^8$ bp and the map length is 600 cM (Schwarz-Sommer et al., 2003). The theoretical relation between the genetical and physical distance is thus 600 kb/cM ($3.6x10^8$ bp/600 cM). "marker e33" mapping at 0.23 cM from *FIS* was then calculated 138 kb far from the *FIS* locus, which is a reasonable distance to start chromosome walking. Both ends of BAC 53b14 insert were used to proceed with chromosome walking (Appendix 6.3 and 6.4 and Figure 3.10), as initially it was not clear which end is proximal and which is distal to *FIS*. Screening the library with the 53b14-T3 end yielded two BAC clones, 74b15 and 40g15, but screening the library with the T7 end of the resulting contig (e.g. 74b15-T7) did not yield new clones. This genomic region is thus not represented in the BAC library and chromosome walking from this side was therefore not possible.

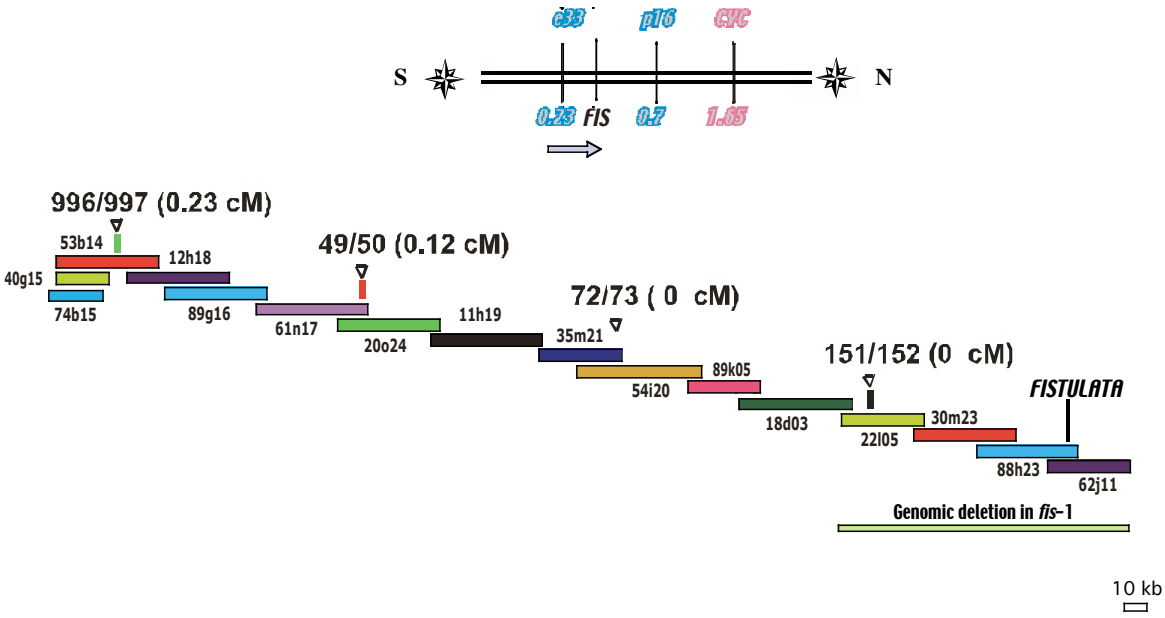


Figure 3.10: Schematic diagram of the BAC contig identified by chromosome walking. The upper panel is a close up of Lg 6 with the new molecular markers tightly linked to *FIS*. The arrow points the genomic region that has been analyzed by chromosome walking. The *FIS* genomic contig is schematically represented in the diagram below. BAC clones are represented by colored horizontal bars whose names are indicated. Molecular markers are shown together with their genetic distance to *FIS*. Vertical colored lines indicate markers generated from polymophisms detected in ESTs contained by the relative BAC clones (Table 6.3 and 6.5). *FIS* is located in a ~ 200 kb genomic deletion of the *fis*-1 mutant.

Walking with the 53b14-T7 end generated a CAPS marker (49/50) which mapped at 0.12 cM (e.g. 66 kb) from *FIS (*Figure 3.9 and 3.10) suggesting that the 53b14-T7 end is proximal to *FIS*. We actually spanned at least 580 kb to get molecular markers fully co-segregating with the *fis* phenotype (marker 72/73, Figure 3.9 and 3.10) and additional ~400 kb to land in the BAC clone where *FIS* is located. The estimated relation between genetic and physical distance is thus higher in this genomic region than expected. Still, our findings confirm that chromosome walking with the available tools is feasible in Antirrhinum.

Interestingly, we found a genomic deletion of ~ 200 kb (spanning 4 BACs) co-segregating with the *fis* character (marker 151/152; Figure 3.9 and 3.10). This deletion involves at least 7 expressed genes (Appendix 6.5). It will be perhaps interesting to pursue in the future the intriguing fact that deletion of hundreds of kilo bases of the genome including transcribed regions does not obviously affect development other that a peculiar defect in the flower.

3.3 Isolation of the *FIS* genomic locus

Final identification of the *FIS* gene was performed in collaboration with Michiel Vandenbussche and Tom Gerats (Radboud University, Nijmegen). This cooperation was prompted by the phenotypic similarity of the *bl* and *fis* mutants (Figure 3.2) indicating that the respective wild-type genes share functional similarity. Successful identification of the *BL* gene revealed its relation to the *MIR169* gene family (Reinhart et al., 2002). A primer based on the sequence of miR169 (MIBUS174) was then derived for a Genome Walker approach in Antirrhinum (described in 2.13). Genome Walker PCR generated several products for each restriction-adaptor ligated library (Figure 3.11) and cross hybridization with individual fragments identified five independent PCR fragments (1a, 1b, 2c, 2d, 5e in Figure 3.11).

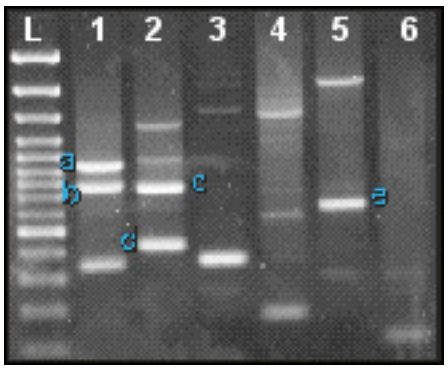


Figure 3.11: Identification of miR169 containing genomic regions by Genome Walker. PCR was performed on six restriction adapter ligated libraries (in lanes 1 to 6 *DraI*, *EcoRV*, *PvuII*, *StuI*, *HincII*, and *NaeI*, respectively) using the miRNA 169 core sequence as the specific primer for PCR. Southern hybridization with individual fragments identified five independent PCR fragments indicated with letters in the photograph that shows the fragments in each PCR reaction after agarose gel electrophoresis.

To analyze if one of these fragments corresponds to the *FIS* locus, southern blots with Antirrhinum wild type and *fis* mutant DNA were prepared and hybridized to individual fragments. The rational of this experiment was to detect differences in the hybridization pattern between the wild type and *fis* mutants for fragments that originated from the *FIS* genomic region. Because *fis*-1 is a deletion mutant, we expected a lack of hybridization signal and because *fis*-2 is an unstable mutant, we expected size differences between the mutant and its homozygote wild-type revertant.

Hybridization with the "1b fragment" fulfilled our criteria (Figure 3.12): both wild types and mutants shared a fragment of 4 kb size, but a ~5 kb wild-type fragment was absent in the *fis*-1 mutant. In *fis*-2 a smaller fragment appeared whose size was shifted in the revertant, although the size of the wild-type fragment was not

restored. Subsequent analysis of revertant alleles revealed, that in this revertant the insertion site of the transposon was not precisely restored upon excision and therefore the *HindIII* restriction site used for digestion of the genomic DNA in the southern detection experiment was lost.

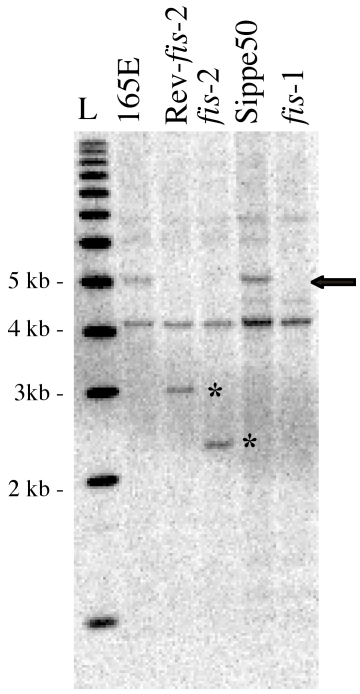


Figure 3.12: "1b fragment" detects the *FIS* gene by southern hybridization. Genomic DNA from plants whose genotypes are indicated above the slots was digested with *HindIII* and loaded onto an agarose gel. The size ladder (L) is shown at the left. After electrophoresis and blotting the membrane was hybridized with "fragment 1b" (Figure 3.11). Arrowhead points to wild-type fragments and the deletion in *fis*-1. Stars show fragments in the *fis*-2 mutant and revertant (rev.). Notice size shift of the fragment in the *fis*-2 revertant.

"1b fragment" hybridized to BACs 88h23 and 62j11 confirming that the *FIS* gene is located within the large deletion previously identified in the *fis*-1 genome (Figure 3.10). The 5 kb *HindIII* fragment of the BAC clones that hybridized to the "1b fragment" was subcloned and sequenced. The sequence of *FIS* is deposited in the EMBL Nucleotide Sequence Database under the accession number AM422776.
Ultimate proof for locus identity was achieved by sequencing PCR-amplified genomic regions from ten wild-type revertants of the genetically unstable *fis*-2 allele (Dr. R. Castillo, personal communication).

3.4 *FIS* as a microRNA:structure, biogenesis and expression

FISTULATA in Antirrhinum and *BLIND* in Petunia are thus related since *fis* and *bl* are due to a mutation in the same gene family encoding the miR169. The pre-miRNA secondary structure was predicted using the RNAfold program (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi): the stem-and-loop hairpin

structure is ~166 bp in length, contains 8 small symmetric bulges and the 21 nucleotides long microRNA at the 5 side of the stem. The overall structure is conserved between pre-miRFIS and pre-miRBL, although the nucleotide sequences differ (Figure 3.13).

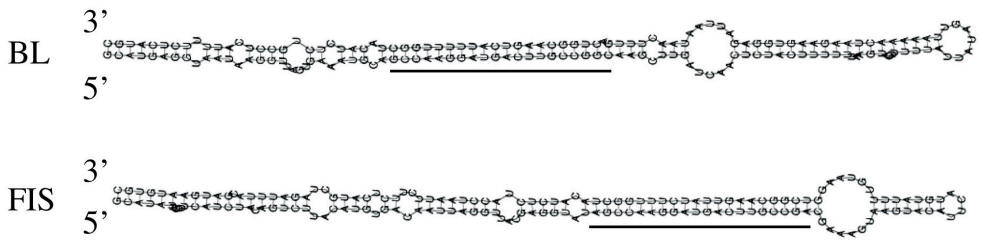


Figure 3.13: Stem-and-loop structure of the FIS and BL pre-miRNAs. Secondary structure calculations were done by the RNAfold program (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi). The stem loop is ca. 166 bp long and contains 8 small symmetric bulges. The predicted structure is conserved between *FIS* in Antirrhinum and *BL* in Petunia. The vertical line shows the position of the mature miRNA at the 5 side of the stem.

The 18 nucleotides long microRNA core sequence is conserved between Antirrhinum, Petunia and Arabidopsis, but the sequences flanking the core differ: miRFIS is not related to any of the 14 AtmiR169 sequences, whilst miRBL is related to the AtmiR169 b-c class (Figure 3.14).

AtmiR169a 5'–C**AGCCAAGGAUGACUUGCC**GA–3'
AtmiR169b-c C**AGCCAAGGAUGACUUGCC**GG
AtmiR169d-g UG**AGCCAAGGAUGACUUGCC**G
AtmiR169h-n U**AGCCAAGGAUGACUUGCC**UG

miRBL C**AGCCAAGGAUGACUUGCC**GG
miRFIS U**AGCCAAGGAUGACUUGCC**GA

Figure 3.14: Sequence alignment of the mature AtmiR169 (from a to n), *BL* and *FIS* miRNAs. The microRNA core (in black) is conserved but nucleotides at the boundaries (in red) can differ.

3.4.1 Expression analysis of *MIRFIS*

The 5' end of pri-miRFIS was determined by 5' RACE using 0330 gene specific spanning the microRNA (Appendix 6.6) but the 3' end of the transcript could not be detected by 3' RACE (Figure 3.15A).

5´RACE products were sequenced and primer 0555 was derived to perform semi-quantitative RT-PCR analysis in combination with primer 0330 (Appendix 6.6). SQ-PCR showed that pri-miRFIS is expressed in all four types of floral organs in young stage 2 flowers, with higher expression in petals and stamens compared to sepals and carpels (Figure 3.15B).

These findings support the genetic studies that point to a role of *FISTULATA* in the inner whorls (3.1.7), rather than to a region specific repressor role in the outer whorls.

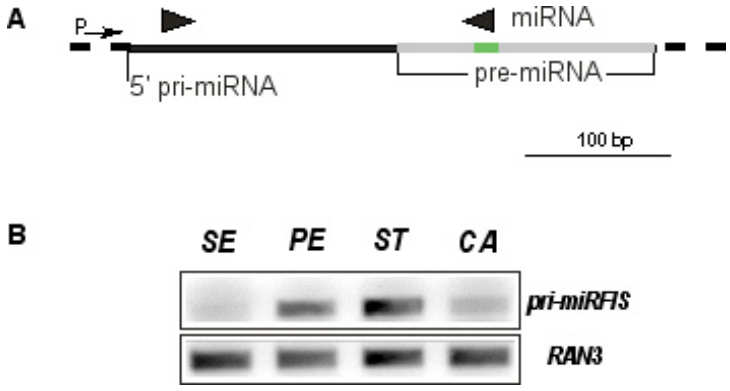


Figure 3.15: Schematic representation of the *FISTULATA* gene (A) and expression of pri-miRFIS in dissected floral organs (B). **A**. The 5' end of the pri-miRNA as detected by 5'RACE is shown by a vertical line and the grey bar shows the pre-miRNA region as predicted by the RNAfold program (Figure 3.13). The mature microRNA is shown in green. Staggered lines indicate the promoter (P) and 3' regions of the transcript. The arrow indicates the direction of transcription and arrowheads show the position of the primers used for RT-PCR analysis. The size scale is indicated by the horizontal bar. **B**. Semi-quantitative RT-PCR to detect pri-miRFIS expression in sepals (SE), petals (PE), stamens (ST), carpels (CA). RAN3 was used as internal control. In situ analysis using Locked Nucleic Acid (LNA) DIG-labelled oligonucleotides as anti-sense probes confirmed the ubiquitous expression of *MIRFIS*. (Dr. N. Efremova, personal communication).

3.5 *NF-YA*s as targets of miR169

AtNF-YAs were experimentally identified as targets of the miR169 (Rhoades et al., 2002) containing a microRNA-recognition-element (MRE) (Kiriakidou et al., 2004) in their 3' UTR. Since the ability of microRNAs to recognize target mRNAs is conserved between species (Ambros et al., 2003) we decided to clone Antirrhinum NF-YAs and test whether their expression is regulated by miRFIS.

3.5.1 Cloning of the Antirrhinum *NF-YA*s

We isolated six Antirrhinum NF-YAs (named AmYA1 to AmYA6); AmYA1, AmYA3 and AmYA4 were already partly represented in the EST library and the three others were detected by their sequence similarity to the *AtNF-YAs*. For this purpose probes derived from Arabidopsis were used for heterologous screening of an *Antirrhinum* phage cDNA library (2.14). In addition, cDNA variants differing within their 5' untranslated region were also detected for AmYA2 and AmYA4. These variants result from alternative splicing as revealed by sequencing the respective genomic regions (Figure 3.16).

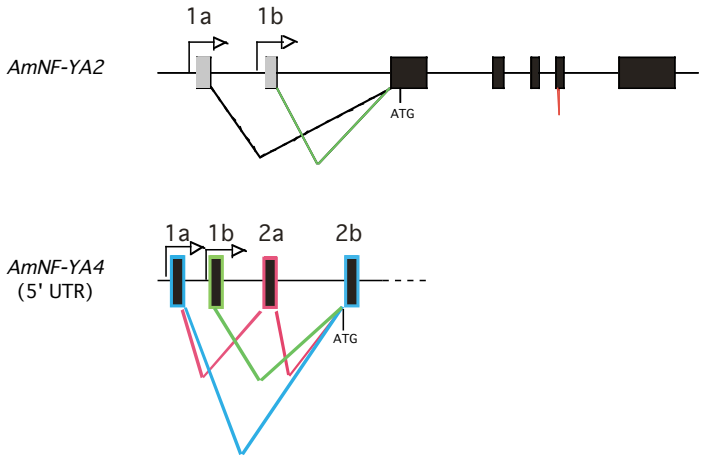


Figure 3.16: Schematic diagram of *AmYA2* gene and *AmYA4* 5' UTR genomic region. Exons are represented by boxes. The products of alternative splicing are indicated by lines in different color connecting the respective exons. Three splicing variants were detected for AmNF-YA2: two of them are likely generated by transcription from alternative promoters and differ for their first exon (exon 1a and 1b, respectively), but they share the same protein coding region. The third splicing variant results in a deletion of 3 amino acid (indicated by a redlines) at the beginning of the exon 5. The lower drawing shows the *AmNF-YA4* genomic region relevant for alternative splicing. Three splicing variants were detected for this gene: two of them can be generated by transcription from alternative promoters and differ in their first exon (exon 1a and exon 1b), similar to *AmNF-YA2*. The third variant contains exon a, an alternative second exon (exon 2a) and exon 2b. The three variants share the protein coding region. The ATG start codon of translation is indicated.

Alternative splicing is not unusual of NF-YAs in plants (Albani, 1995) or in animals and likely contributes to the regulation of their expression by affecting translation of the gene products (Mantovani, 1999). We confirmed the expression of the splice variants by RT-PCR (not shown), but the biological relevance of alternative splicing was not studied in detail.

The AmYA cDNA sequences are deposited in the EMBL Nucleotide Sequence Database under the accession numbers AM422770-AM42277

3.5.2 Evidence for target regulation by miRFIS

MiRNAs recognize their targets by base pairing at the MRE site. For the efficiency of this process in plants the following empirical parameters were established (Schwab et al., 2005). Counting the nucleotides from the 5´of the microRNA:

1. No more than 1 mismatch within position 2-12
2. G:U pairs are considered as 0.5 mismatches
3. No mismatch at position 10 and 11.
4. No more than 2 consecutive mismatches downstream of position 13.

Based on these criteria, four Antirrhinum NF-YAs (AmYA1 to AmYA4) contain a MRE within their 3' UTR that is sensitive to miRNA-directed regulation (Figure 3.17). The other two NF-YAs (AmYA5 and Am YA6) do not contain a recognition site.

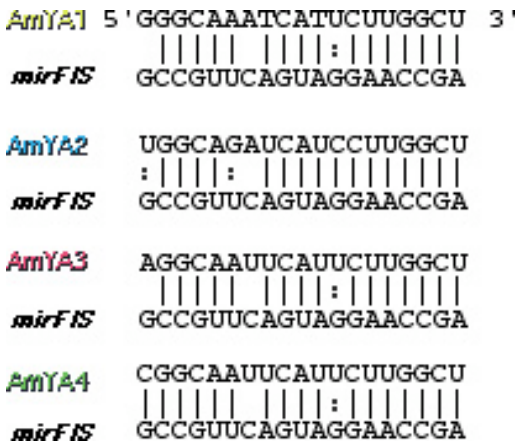


Figure 3.17: Coplementarity between the miRNA-recognition elements present in the 3' UTR of AmNF-YAs and miRFIS. The numbering of base pairs starts at the 5' of miRFIS. G-U mismatches are indicated by ':' .Four AmYAs contain a MRE and are equally sensitive to miRFIS recognition (Schwab et al., 2005).

We used an *Agrobacterium*-mediated transient expression system in *Nicotiana benthamiana* to study the role of miRFIS in the recognition of NF-YAs as targets (Llave et al., 2002). For this purpose, ~ 91 bp of the AmYA2 3' UTR containing the MRE was fused C-terminal to the Yellow Fluorescent Protein (YFP) coding sequence (35S::YFP-YA2wt) and co-infiltrated in tobacco leaves with a construct carrying 35S::miRFIS. The rational was to compare YFP fluorescence after infiltration of the YFP construct in the absence and presence of miRFIS, expecting lower signal intensity in the latter case. As a control, a second construct with an altered MRE

site (35S::YFP-YA2mt) was prepared. Base pairing with miRFIS was interrupted between positions 8 and 13 (positon 1 at the putative 5' end of miRFIS; Figure 3.18A). A more precise interference with miRNA recognition was not possible, because it is yet not the site of the potential cleavage.

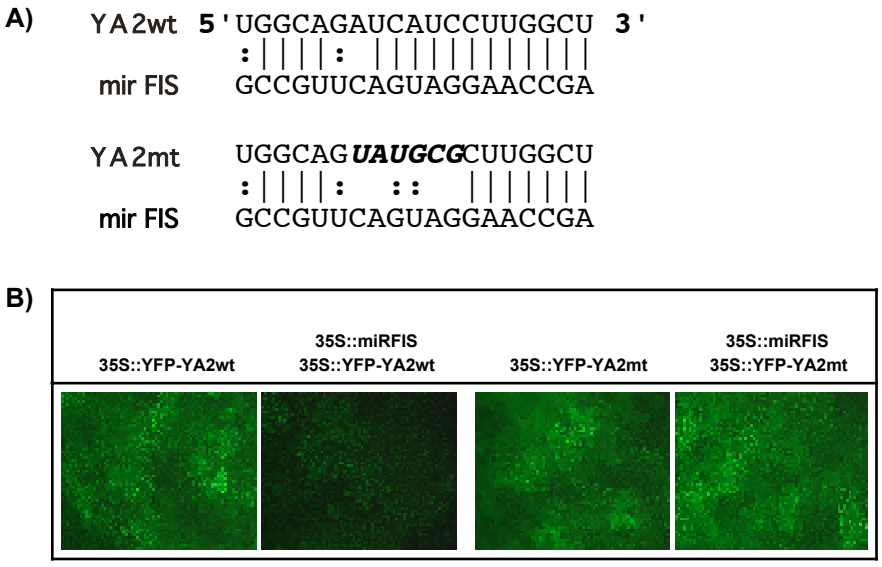


Figure 3.18: *Agrobacterium*-mediated transient expression in *N. benthamiana*. A) Alignment of the wild-type and mutant AmYA2 MREs with miRFIS. The stretch of 6 altered nucleotides is highlighted in bold and italic style. B) YFP fluorescence decreases when YFP-AmYA2wt is coinfiltrated with miRFIS. Co-infiltration of miRFIS with YFP-AmYA2mt does not affect the signal intensity. Infiltration was performed in a 1:8 target/miRFIS ratio. The constructs used for infiltration are indicated above the panel.

First experiments with this system suggest that the AmYA2 MRE is recognized by miRFIS (Figure 3.18B). However, the target/miRNA ratio as well as the density of the bacterial suspension are crucial for reproducibility in these experiments, an observation which is not mentioned in relevant publications. Accordingly, a target/miRNA ratio higher than 1:8 results in the recognition of the mutant MRE and if the ratio is lower, then the wild-type MRE is not longer recognized. Analyses to determine the mRNA and protein levels in the infiltrated leaves are in progress to learn whether miRFIS-mediated regulation occurs by mRNA cleavage and/or translational inhibition (see 1.2.3).

3.6 *NF-YA* expression in the flower

The NF-Y protein complex binds to the CCAAT DNA regulatory element (see 1.3). Two such CCAAT boxes are present in the second large intron of *PLE* and *FAR*

genes (Davies et al., 1999) adjacent to several others cis-acting regulatory elements that control the spatial and temporal expression of the C- genes in the flower (Figure 3.4a). A recent study showed that the CCAAT motif is conserved within the second intron of the class C- genes of several species (Hong et al., 2003), suggesting a possible role for this sequence in the positive control of their expression. It appears likely therefore, that miRFIS controls *PLE* and *FAR* by negative regulation of the NF-YAs.

As shown in Figure 3.19, AmYAs are ubiquitously expressed at very low level in both vegetative and reproductive tissues and during all tested stages of flower development (e.g. from inflorescences up to 5 mm to flower 2-2.5 cm in size).

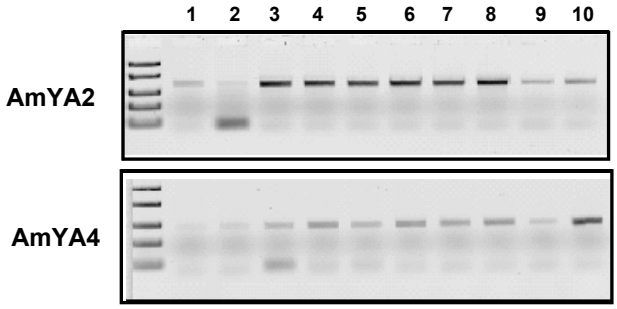


Figure 3.19: Ubiquitous expression of AmYA2 and AmYA4 in various Antirrhinum tissues. Qualitative RT-PCR was performed with RNA from 1) 14 days total seedlings with root, 2) 28 days old total plants, 3) 42 days old total plants, 4) Inflorescences up to 5mm, 5) Buds 7-8 mm with visible petals, 6) Buds 10 mm, 7) Flowers 2-2.5 cm, 8) Unfertilized carpels, 9) Mature flowers, 10) Fertilized carpels.

Possible changes in AmYA transcript abundance were analyzed by qRTPCR comparing wild-type and mutant flowers at early and late developmental stages. As described in 2.16, the plant material was pooled after harvest to minimize the biological variance and three technical replicas were prepared for each genotype. Due to the lack of biological replicas thorough statistical evaluation of the observed values could not be performed.

3.6.1 Early developmental stages

To analyze NF-YAs expression at early stages we collected 3-5 mm long wild type and *fis* inflorescences. qRTPCR analysis did not detect clear changes in the expression of the NF-YAs containing the MRE at their 3´UTR (e.g. NF-YA1 to NF-YA4), while reduced mRNA levels could be detected for NF-YA5 and NF-YA6 in *fis* inflorescences (Figure 3.20). Several possibilities can explain why the NF-YAs

containing the MRE do not show changes in transcript abundance when comparing wild-type and mutant inflorescences. First, the analyzed tissue contained a mixture of flowers at different developmental stages along with bracts and the inflorescence stem, thus changes at a particular stage of flower development would escape detection due to low representation in the mass of other tissues. The fact that we could not detect change in PLE/FAR expression points to this dilution problem (Figure 3.20), because the phenotype of the *fis* mutant clearly indicates their enhanced expression. Second, it is possible, that *FIS* regulates NF-YA in a 'tuning mode', where changes in expression are small and difficult to detect. This can be further complicated by the fact that microRNAs can regulate gene expression at the level of translation. Third, miRFIS regulation might not have relevance for NF-YA or C-gene expression during early stages of flower development. This latter option is contradicted by observing enhanced PLE expression during early flower development in *fis* mutants by in situ hybridization (Dr. N. Efremova, personal communication).

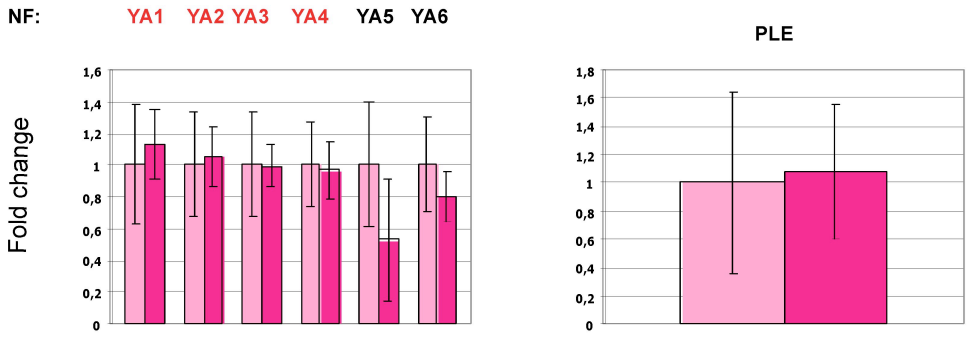


Figure 3.20: qRTPCR analysis of NF-YA and PLE expression in wild type and *fis* inflorescences. The chart in the left panel shows the change in expression in the *fis* mutant inflorescences (dark color) relative to the wild type (light color). NF-YAs containing the MRE (AmYA1 to AmYA4) are indicated in red at the top. The chart in the right panel shows the change in expression of PLE in *fis* mutants as compared to the wild type. For all calculations expression in the wild type was set to the value of 1. Error bars represent standard deviation based on three technical replicates, the strong variation is due to low expression levels preventing reproducible amplification.

3.6.2 Late developmental stages

To analyze expression of the NF-YAs in the four floral organs, we collected flowers at stage 2 of wild type and *fis* mutants when floral organs are large enough to carefully dissect them. We expected to see NF-YA expression in the inner whorls of wild type flowers where they positively regulate the C-genes, while expansion of expression to the outer whorls was expected in *fis* flowers.

NF-YAs are expressed in all floral organs of the wild type flower, although the level and organ-specific pattern of expression slightly differs comparing different NF-YA genes. NF-YA5 and NF-YA6 are expressed at high levels in all whorls, perhaps due to the lack of miRFIS-mediated post-transcriptional regulation, while expression of NF-YA1 to NF-YA4 is fairly low in all whorls. As expected, expression of PLE and FAR is low in the perianth organs whilst they are expressed at higher levels in the inner whorls (Figure 3.21).

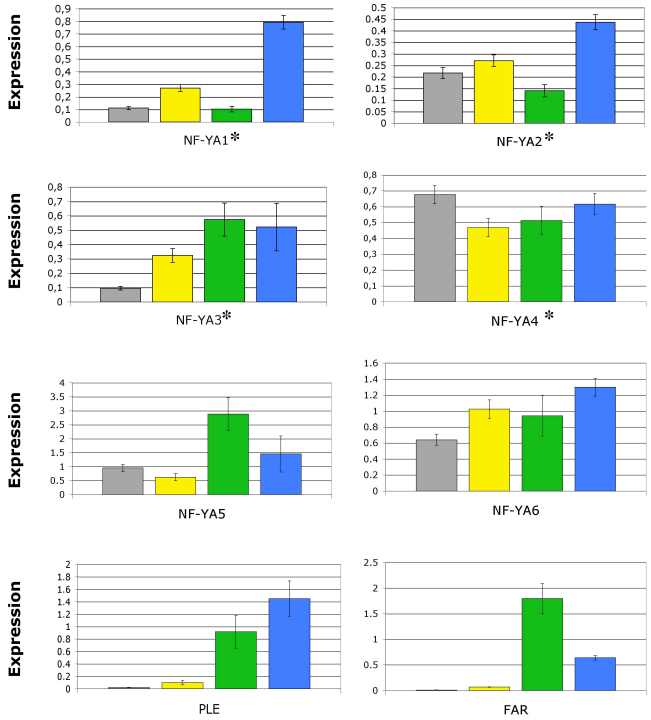


Figure 3.21: NF-YA and PLE expression in dissected floral organs. Wild type flowers at stage 2 were collected and the four floral organs dissected. qRT-PCR analysis was performed in sepals (indicated in grey), petals (yellow), stamens (green) and carpels (blue). The detected NF-YA levels were normalized to actin and GAPDH. NF-YAs containing the MRE are highlighted with an asterisk. Error bars represent standard deviation based on three technical replicates. Ubiquitous expression of of the NF-YAs was confirmed by in situ hybridization of young Antirrhinum inflorescences (N. Efremova, personal communication).

Expression of NF-YA1, NF-YA2 and NF-YA3 and NF-YA4 was enhanced in one or the other (or all) organs of *fis* flowers as compared to the wild type (Figure 3.22). NF-YA6 expression in *fis* mutant flowers does not appear to be affected, but intriguingly, NF-YA5 was down-regulated in the inner whorls. Downregulation of NF-YA5 in stamens and carpels of the *fis* mutant is possibly a compensatory effect due to overall enhancement by cumulative changes in expression of the other, MRE-containing NF-YAs, but this speculation has not been further pursued.

In agreement with our expectation PLE is upregulated in the outer whorls of *fis*, but in the inner organs its expression seems to be lower than in the wild type. There are two possible explanations for this observation. First, decreased PLE expression in the *fis* inner whorls can be possibly ascribed to differences in the developmental stage of the collected wild type and *fis* material. PLE expression in the inner whorls is indeed known to decrease after stage 7 by becoming confined to specialized structures within the anthers and carpels (Davies et al., 1999). However, although it is difficult to harvest uniform plant material at exactly the same developmental stage, it appears unlikely that this inaccuracy affects mutant flowers only; in addition the material collected for this analysis is far older than stage 7. Second, NF-YA5 down-regulation observed in the inner whorls of *fis* flowers might be correlated to decreased PLE levels in stamens and carpels at late developmental stages in differentiated organs, an option which will need to be considered in the future.

3.6.3 Conclusions

qRT-PCR analysis was used in this study to determine the effect of miRFIS on NF-YA expression in particular for MRE-containing genes. The following conclusions can be drawn. First, MRE-containing NF-YAs are expressed at a very low level in the flower while non MRE-containing NF-YAs show higher expression levels. Second, changes in NF-YA expression in *fis* mutants became detectable when comparing dissected organs representing flowers already advanced in development, but sampling problems prevented to obtain reliable results with young flower buds. qRT-PCR is therefore a limited method for the analysis of early effects of miRFIS-mediated regulation. In situ hybridization of young Antirrhinum inflorescences using NF-YAs as anti-sense probes (performed by Dr. N. Efremova) appeared therefore a more appealing tool, but results of this analysis are only briefly mentioned in this report. Third, pri-miRFIS and the NF-YAs seem to have an overlapping expression pattern in the flower supporting the hypothesis of a fine-tuning mechanism of NF-YA regulation by the microRNA.

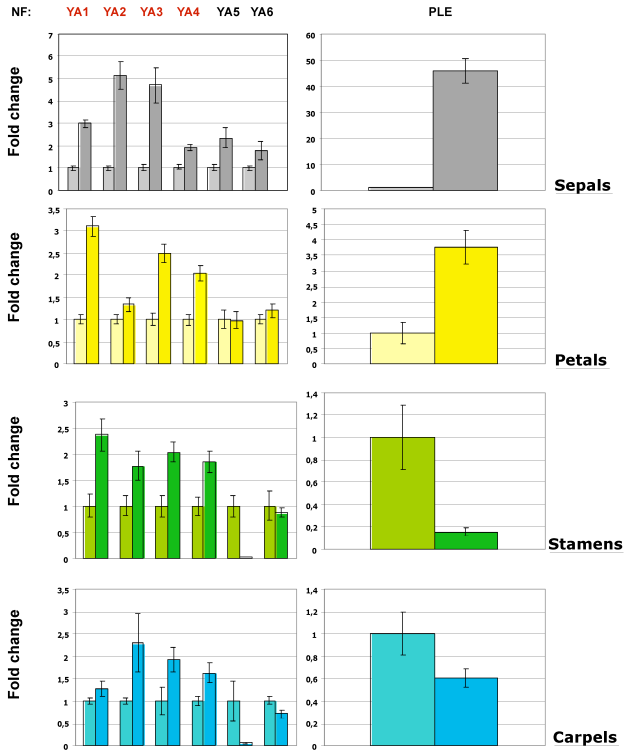


Figure 3.22: Q-PCR analysis of NF-YA and PLE expression in wild type and *fis* flowers. Flowers were collected at stage 2 and the four floral organs dissected: sepals (grey), petals (yellow), stamens (green), carpels (blue). Fold change expression of the NF-YAs in *fis* floral organs as compared to the wild type (lighter colored histogram, see also Figure 3.20) is represented in the left panel. Fold change expression in the single organs appears higher than when looking at entire inflorescences (Figure 3.20), but is not higher than 3 fold. NF-YAs containing the MRE are indicated in red. In the right panel the expression of PLE in the *fis* flower as compared to the wild type is shown. Their expression in the *fis* mutants is higher in the perianth as compared to the wild type. Error bars represent standard deviation based on three technical replicates.

4. DISCUSSION

4.1 Chromosome walking in *Antirrhinum majus*

A genomic BAC library established in our laboratory allowed us to initiate cloning of the *FISTULATA* gene by chromosome walking. Map-based cloning then was combined with a candidate gene approach and a locus identity test utilizing wild-type revertants of the genetically unstable *fis*-2 allele. Problems and advances encountered during this first map-based approach to clone an Antirrhinum gene can be summarized as follows.

1. The relation of 600 kb/cM between physical and genetic distance calculated from linkage analysis and genome size (Schwarz-Sommer et al., 2003) turned out to be underestimated in the *FIS* region; according to our observations this ratio was six times higher (3.2.4). Whether this represents a local or general disagreement of the calculation between the theoretical and experimental distance needs to be proven in the future.

2. The calculated seven–fold coverage of the Antirrhinum genome by the BAC library was insufficient to evenly represent the genome resulting in a failure to detect BAC clones 'South' from "marker e33". Fortunately, this did not cause difficulties in our project, because this direction was distant to the *FIS* locus (Fig. 3.10), but the problem has to be solved in the future by preparing additional BAC libraries, preferably by using a different restriction enzyme to generate genomic fragments.

3. The frequent presence of repetitive sequences at the BAC ends creates a problem for chromosome walking. We could overcome this problem by screening of the Antirrhinum EST library with BAC inserts of interest. This strategy provided us with single copy sequences, which could be used for the detection of adjacent BAC clones as well as with useful information for deriving CAPS markers for mapping the BAC contigs. This strategy is therefore an appealing complementary tool to be applied for chromosome walking for species whose genome has not yet been sequenced.

4.2 *MIRFIS* in the spatial control of C-gene expression

Molecular cloning of *FIS* (3.2-3.4) revealed that the gene encodes a bona fide microRNA, related to miRBL in Petunia and to members of the miR169 family. The targets of miR169-mediated post-transcriptional regulation are members of the *NF-YA* transcription factor gene family (1.3), which we cloned from Antirrhinum and analysed their expression (3.6). Molecular studies supported by genetic observations (3.1) allowed us to propose a dynamic rather than spatial model for the regulation of the C-function by miRFIS, where primary patterning of C-gene expression is due to spatially controlled activation. Experimentally determined components of this model, theoretical considerations supporting it as well as experiments to corroborate mechanistic details in the future will be discussed here.

4.2.1 Functional evidence of miRFIS expression in the centre of the flower

Fis mutants display homeotic defects in the outer whorls of the flower (3.1.2) due to ectopic *PLE* expression (McSteen et al., 1998; Motte et al., 1998) suggesting that FIS acts as a repressor of the C-function in the wild-type perianth (Figure 1.4). In contrast to this simple scenario, expression analyses by semi quantitative RT-PCR in dissected floral organs of young flowers revealed that pri-miRFIS is ubiquitously expressed (Figure 3.15). In situ hybridization experiments with DIG-labeled oligonucleotides as anti-sense probes confirmed uniform expression of *MIRFIS* in the inflorescence meristem and in young flowers throughout early development (Dr. N. Efremova, personal communication).

Whether expression of the *MIRFIS* gene fully corresponds to the pattern of miRFIS activity is not yet clear, because we have no information on the spatial distribution of components of the machinery, such as that of the RISC complex, necessary to realize its function. However, genetic studies confirmed the functionality of miRFIS in the center of the young flower, because low level of *PLE*, whose expression is the indirect target of miRFIS, became de-repressed in a *fis* background leading to restoration of stamen and carpel identity in mutants carrying 'leaky' *ple* alleles (3.1.3 and 3.1.4). Floral determinacy was not rescued in *fis ple* double mutants suggesting that the level of PLE function required for this feature is higher than

that for the control of organ identity. Similarly, Arabidopsis plants show quantitative differences in the AG function necessary to prevent floral indeterminacy and for reproductive organ identity (Mizukami and Ma, 1995).

In summary, *FIS* is expressed in the centre of the flower where it controls the level of C-gene expression as also indicated by enhanced *PLE* expression in *fis* mutants by in situ analyses (Dr. N. Efremova, personal communication). Intriguingly, this activity has no consequences for reproductive development as *fis* mutants do not display defects in this respect.

4.2.2 *MIRFIS* tunes, but does not clear expression of its direct targets

The targets of post-transcriptional regulation by miRFIS are NF-YA transcription factors. Four of them contain a MRE in their 3' UTR and preliminary results using a reporter assay in *N. benthamiana* confirm the miRFIS-mediated AmYAs regulation (3.5.2). The currently known Antirrhinum NF-YAs are expressed in all wild-type floral organs and their transcription can be enhanced to some extent in *fis* mutant floral organs (3.6). In situ hybridization using AmYA2 and AmYA4 as antisense probes corroborated their uniform expression in the flower and did not provide evidence for quantitative or patterning changes of their expression in the *fis* mutant (Dr. N. Efremova, personal communication), leaving open the option for translational inhibition.

The consequence of post-transcriptional regulation by miRNAs for target gene expression can be achieved by two alternative mechanisms (1.2.3). In the "clearance model" the miRNA clears the cells from undesired transcripts and in "the fine-tuning model" the steady-state level of the target mRNAs is regulated The result of the first kind of regulation is non-overlapping expression of miRNA and target, primarily based on spatial patterning of miRNA expression. In the second model the miRNAs and their targets need to be co-expressed (so-called incoherent co-expression). Spatially overlapping expression of miRFIS and the AmYAs in the flower meristem thus suggest that miRFIS acts in a tuning mode to adjust NF-YA abundance to a desired level during flower development. It is likely that feed-back loops between the miRNA and the NF-YAs and/or between the AmYAs also contribute to dampening target gene expression, as described for other miRNAs (see 1.2.4), but this needs to be corroborated in the future.

How can the miRFIS/AmYA module exert a regulatory effect on C-gene expression in Antirrhinum? Circumstantial evidence based on phylogenetic considerations suggests that a cis-regulatory element present in the second intron of C-genes in several species serves as the binding site for NF-YA (1.3 and 3.6). Reporter gene fusion studies utilizing these elements in Arabidopsis support a role of the CCAAT-motif in the positive control of AG expression (Hong et al., 2003). Thus it is possible that one or more of the NF-YAs convey the miRFIS-mediated tuning control to the C-genes in Antirrhinum.

4.2.3 *MIRFIS* is an indirect target of *FIM*-mediated protein degradation

As shown in Figure 3.4a cis-acting elements flanking the CCAAT boxes present in the AG intron are also conserved in Antirrhinum and in several other species (Hong et al., 2003), although the distance between these elements and their position can vary. The binding sites for *LFY* and *WUS* in Arabidopsis serve for the flower-specific activation of *AG* in a small region in the centre of the floral meristem where expression of these two transcription factors overlaps (Lohmann et al., 2001; Mayer et al., 1998; Weigel et al., 1992). The precise function of *FLO/ROA*, the Antirrhinum counterparts of *LFY/WUS* in activation of the C-function is not supported by experimental evidence, but the role of WUS in stem-cell maintenance and the respective localised expression are conserved in Antirrhinum as shown by studies with the *ROA* gene and its mutants (Kieffer et al., 2006). This and the presence of a putative ROA binding site in the *PLE* intron suggests that the link between stem cell maintenance and C-gene regulation can be conserved as well. Subsequent to early activation C-gene expression is likely maintained by an autoregulatory mechanism (Gomez-Mena et al., 2005) and is possibly supported by positive control exerted by the NF-YAs (Figure 4.1A). However, if these assumptions are correct then region-specific activation and autoregulatory maintenance of the C-function will coincide with negative regulation by miRFIS. It follows that mechanisms exists which help to overcome this negative control and support early manifestation of the C-function.

As mentioned before (1.1.3.2), *FIM* encodes an F-box protein likely involved in the targeting of repressors of the B- and C- function (Ingram et al., 1997). The defects regarding delayed C-gene expression observed in *fim* mutants were overcome in the *fis* mutant background (Figure 3.6); this epistatic relation of *fis*

to *fim* suggests that *FIS* is an indirect target of FIM-mediated proteolytic degradation. FIM is transiently expressed in the centre of the flower before activation of the organ identity genes (Simon et al., 1994), and thus could target one of the components needed for miRFIS biogenesis and activity to allow proper manifestation of *PLE* expression. Subsequently, when the C-expressing region is free from FIM expression, the miRFIS/NF-YA regulatory module will contribute to the homeostatis of *PLE* expression as outlined in 4.2.4.

Several experimental approaches can be used to clarify details of the control of *FIS* by FIM. Firstly, it would be interesting to see whether the negative effect of FIM on FIS can be recapitulated outside of the flower. For this purpose, *Agrobacterium*-mediated infiltration of *N*. *benthamiana* leaves offers an option; once the effect of miRFIS on the expression of a reporter gene carrying the MRE can be reliably monitored (3.5.2) the effect of concomitant co-expression of FIM can be studied. Secondly, a miRFIS probe could be used for Northern hybridization on RNA isolated from wild-type and *fim* inflorescences (or likewise from an appropriate set-up with infiltrated *N*. *benthamiana* leaves) to discriminate between FIM-mediated effects on different steps of microRNA processing and accumulation.

4.2.4 Mechanism of miRFIS maintanance of the C- genes boundaries

MiRFIS controls the level of C-gene expression in the inner whorls (4.2.1) and this control is mediated by a tuning effect on NF-YAs (4.2.2), but how can the loss of this control in the *fis* mutant result in ectopic expansion of the C-function?

A hint that the level of C-function in the internal whorls is indeed crucial for its ectopic expansion comes from the observation of the dosage effect of *FIS* in the *fim* background: second whorl homeotic changes are absent in plants heterozygote for *FIS* in a *fim* background, although carpel identity in the centre is restored (Figure 3.6). In other words, cells where C-expression is activated somehow transmit this 'signal', likely the gene products themselves, to neighboring cells. C-gene expression can be maintained by the autoregulatory mechanism, provided that a threshold C-activity is reached in these cells. By this means a laterally extending gradient can form and the organogenic potential for reproductive development will manifest itself depending, again, on threshold C-function values necessary to be reached for this function (Figure 4.1B).

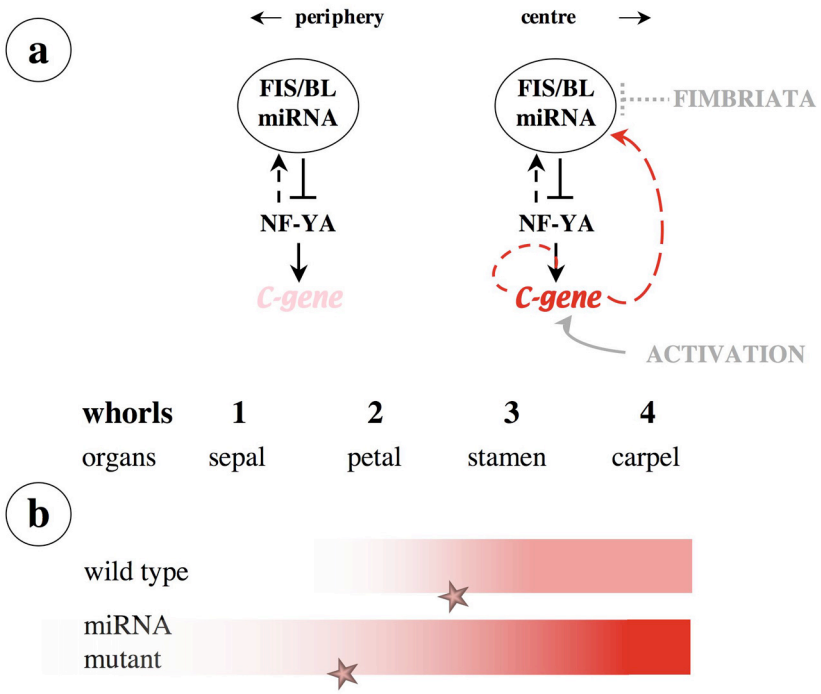


Figure 4.1: Regulatory mechanism governed by miRFIS to maintain the C–domain boundary by controlling the C-expression levels. A) The model represents the mechanism during (in grey) and immediately after (in black) C activation. FIMBRIATA is a transiently expressed F-box protein that temporarily antagonises miRFIS. Dotted lines between FIS and the C genes (red) and the NF-YAs (black) indicate postulated feedback loops to fine tune the NF-YA level and to achieve homeostasis for C-gene expression. Arrows indicate activation and barred lines indicate inhibition. The positions of the floral whorls are indicated by numbers with the respective wild-type organs shown under the numbers. B) The bars shaded in red highlight the result of the regulatory circuit in adjusting the C-expression level in the wild type and in the mutants. In the mutants the miRNA-governed fine-tuning control is impaired and early C-gene expression increases primarily within the domain where C-genes are activated. Lateral extension of the domain results from transmitting excess of C-gene products to daughter cells during cell divisions, reinforced by threshold-dependent autoregulation. Stars mark the threshold C-function level, above which C-gene autoregulation takes place and the organ identity control is realized.

One appealing possibility to generate a C-expression gradient is to consider that C-genes are activated at an early stage of flower development when meristematic cells divide and pass their gene products to daughter cells. Progeny cells outside the activation domain can maintain the activated state of C-expression as long as they obtain the amount of C-gene product necessary to establish the autoregulatory circuit. Consequently, the balance between the initial amount of C-gene product in a cell and the rate of cell division will determine the slope of the gradient and thereby determine when and where C-gene expression falls below a threshold value. in the wild type flower this apparently coincides with the outer boundary of the C-domain between the second and the third whorl, but expands

towards the perianth in the *fis* mutant due to the lack of the control of the initial C-expression level (Figure 4.1).

Additional experiments are necessary to test this dynamic model, in particular by challenging the implication that FIS function in the outer whorls has no major contribution to the spatial regulation of C-gene expression. Expressing FIS or BL in the respective mutant backgrounds under the control of a promoter that confines their expression either to the perianth of the flower or to the central region of the floral meristem is a possibility; if the assumed mechanism of FIS/BL function is correct then the wild-type phenotype will be rescued by the latter transgene, but not by the former one. Likewise, artificially enhanced miRFIS expression in the centre of the flower should result in flowers phenocopying *ple* mutants, or else, enhanced expression of AmYAs with modified MREs should phenocopy *fis* mutant flowers.

4.2.5 Open questions

The model shown in Figure 4.1 summarizes the genetic and molecular observations on the regulatory relation between the miRFIS/NF-YA module and C-gene expression. However, important insights into the precise molecular mechanism underlying this control are missing. For instance, it is not clear whether the tuning-effect of miRFIS on NF-YA expression is achieved by NF-YA transcript cleavage or by translational inhibition (or both) or even which (or how many) of the several potential targets are involved. A modified protocol for 5´RACE to isolate the 3´miRNA-guided cleavage products and determine the cleavage site (Wang et al., 2004) was performed and unfortunately not successful, most probably due to the very low levels of the AmYAs in the flower. If our hypothesis is correct, evidences for the molecular mechanism of miRFIS-mediated regulation will come from the transient experiment in *N.bentamiana* where overexpression of the YFP fused to the miRFIS MRE might overcome the limits related to cleavage products detection (3.5.2).

In spite of functional evidence of miRFIS post-transcriptional control of the AmYAs in the centre of the flower, nothing is know about the biological relevance of this mechanism in the outer whorls. The absence of additional phenotypes in the *fis* flowers could be due to redundancy: the presence of miR169 derived from other *MIR169* loci might compensate for the absence of miRFIS. Currently we do not

know how many members of the *MIR169* family are present in Antirrhinum but we do not exclude the possibility that there is more than one since this family is used to be highly redundant within species where it has been characterised. It would be interesting to isolate those by stem loop RT-PCR that allows to amplify individual precursors from cDNA (Chen et al., 2005; Chen, 2004) and analyze individual members by looking for instance at their expression pattern by in situ analysis. One limitation to this will be, however, the lack of mutants in these genes to reliably control the contribution of the observed patterns to possible redundancy.

4.3 Conservation of the *MIRFIS* function across species

The *MIR169* gene family is conserved within monocots and eudicots indicating that the miR169/NF-YA represents a phylogenetically conserved module of gene regulation since the two groups diverged from a common ancestor between 120 and 200 million years ago (MYA) (Fahlgren et al., 2007; Wolfe et al., 1989). Phylogenetic conservation of microRNAs and their targets, however, does not necessarily imply a comparable regulatory role in distantly related species; it rather seems that the modules remain constant, but small changes in their temporal, spatial or environmental regulation can have large impact on the diversification of organisms during evolution (Axtell and Bartel, 2005).

In the case of the miR169-mediated control of C-gene expression, the mechanism is conserved in the Asterids Petunia and Antirrhinum, as evidenced by the similarity of the *bl* and *fis* mutant phenotypes. Components of this control such as AtmiR169, NF-YAs and the respective cis-acting CCAAT element in *AG,* also exist in the Rosid Arabidopsis, but some elements of the early regulatory circuit mediated by miRFIS in Antirrhinum are missing. For instance, while FIM antagonizes *FIS*, UNUSUAL FLORAL ORGAN (UFO), the Arabidopsis orthologue of FIM, does not play any role in the positive control of early *AG* expression (Ingram et al., 1995). It is likely therefore that other mechanisms govern early adjustments of the C-expression level in this species. Such difference in mechanistic details in early control processes in different species is also suggested by the lack of influence of *AP2*-like genes on the C-function in Antirrhinum and Petunia (1.1.3), which indicates a lack of functionality of the miR172/AP2 module (Chen, 2004) in this control. This suggests that functional diversification of

conserved miRNA/target modules took place between higher plant lineages the extent and effect of which will be interesting to pursue in the future.

5. SUMMARY-ZUSAMENFASSUNG

The identity of *Antirrhinum* reproductive organs is controlled by two class C- MADS box genes, *PLENA* (*PLE*) and *FARINELLI* (*FAR*), which are expressed in the third and the fourth whorl of the flower. The *fistulata* mutant (*fis*) in *Antirrhinum* and *blind* (*bl*) in *Petunia* show partial homeotic conversion of petals to stamenoid structures, which is caused by ectopic expression of PLE/FAR and pMADS3/FBP6, the Petunia C-genes, respectively. *FIS* and *BL* thus control the C- expression domain in the wild type. *FISTULATA* was cloned by a map-based strategy combined with a candidate gene approach and show that both genes encode a microRNA corresponding to miR169. The miR169 target site is present in the 3´UTR of members of the NF-YA transcription factor family that bind a cis-acting CCAAT motif present in the second intron of the *PLE*/*FAR* and *pMADS3* genes. This suggests a miRNA-mediated and NF-YA-dependent control of the C- function. We cloned six members of the Antirrhinum NF-YA gene family (AmYAs), four of which contain the miRNA recognition site. Data derived from in vitro infitration experiments indicate miRFIS-dependent post-transcriptional regulation of AmYAs. Expression studies in wild type and *fis* mutant flowers showed that miRFIS and the AmYAs are spatially co-expressed in the flower suggesting that by fine tuning expression of the NF-YAs, miRFIS indirectly regulates the level of C-gene expression. In addition, genetic evidence is presented demonstrating the functional relevance of the unexpected miRFIS function in the centre of the flower. Based on these results a dynamic model is proposed in which miRFIS maintains the boundaries of the C- domain by preventing formation of a radially extending molecular gradient from the centre of the flower (where C-products are activated) toward peripheral regions of the meristem. Experimental approaches are suggested to solve open questions.

Die Identität der reproduktiven Blütenorgane wird in *Antirrhimum* durch die MADS-box-Gene *PLENA* (*PLE*) and *FARINELLI* (*FAR*) kontrolliert. Diese sogenannten Klasse C-Gene sind im dritten und vierten Wirtel der Blüte exprimiert. Eine partielle homeotische Umwandlung von Petalen zu Stamen in der *fistulata* (*fis*) Mutante in *Antirrhinum* und in der *blind* (*bl*) Mutante in *Petunia* ist durch die ektopische Expression der C-Gene bedingt. Hieraus folgt, dass die C-

Expressionsdomäne in der Wildtyp-Blüte durch *FIS* und *BL* kontrolliert ist. Wir haben das *FISTULATA*-Gen über eine, auf Kartierung basierenden (map-mased) Strategie kloniert. Wir konnten zeigen, dass *FIS* und auch *BL* für eine mikroRNA kodieren, die zu der miR169-Famile gehört. Die Sequenz, die durch miR169 erkannt wird, ist in dem 3'-Bereich (3'UTR) von Mitgliedern der NF-YA Familie von Transkriptiosfaktoren vorhanden, die ihrerseits cis-aktive Motive (CCAAT-box) in dem zweiten Intron von C-Genen erkennen. Es ist deshalb wahrscheinlich, dass die miRNA-Kontrolle der C-Gene über NF-YA vermittelt wird. Wir haben sechs Mitglieder der NF-YA Familie in *Antirrhinum* (AmYAs) kloniert und festgestellt, dass vier die miRNA-Erkennungssequenz besitzen. Vorläufige Beobachtungen in einem Infiltrationssystem *in vitro* deuten darauf hin, dass die posttranskriptionelle Regulation von AmYAs tatsächlich über miRFIS erfolgen kann. Analysen der miRFIS und *NF-YA* Expression in Blüten von Wildtyp- und *fis*-Pflanzen zeigten einen räumlichen Überlapp. Hieraus schließen wir, dass die räumliche Regulation der C-Gene durch miRFIS über eine Feinabstimmung der NF-YA-Expression bewerkstelligt wird. Auch genetische Untersuchungen bestätigen die funktionelle Relevanz der unerwarteten miRFIS Expression im Zentrum der Blüte. Wir schlagen auf Grund dieser Ergebnisse ein dynamisches Modell vor, in dem miRFIS das Expressionsniveau der C-Gene im Zentrum der Blüte (wo diese aktiviert werden) unter einem Schwellenwert hält. Ein Überschreiten dieses Niveaus in der Mutante bedingt, dass Tochterzellen während der Zellteilung eine zu hohe Dosis an C-Genprodukten erhalten, wodurch der laterale Gradient von Zellen, die die C-Funktion ausüben können, ausgedehnt wird. Es werden experimentelle Lösungen vorgeschlagen um offene Fragen zu klären.

6. APPENDIX

6.1 Sequences of the BAC insert ends

***A.majus* 40g15-T7**

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***A.majus* 40g15-T3**

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***A.majus* 74b15-T7**

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***A. majus* 74b15-T3**

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***A. majus* 53b14-T3**

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***A. majus* 89g16-T7**

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***A. majus* 61n17-T7**

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***A. majus* 61n17-T3**

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***A. majus* 20o24-T7**

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TTCTATATATAATTATATATGGGGAGACGATGCATCGCCTCTGCAGCATTCTTTTCGGTTCGCTTATTA
TGCAGTTCTATTGCTTGCGGCAGAATGCTTGGTCCGGTGGATATATAGCTGGAACAAGTATTGGTAAAA
TGGCAGCAGCTGCTANTNNNAAGGCGGTAGTTGAATGAAAAGACGTGCTAGTCCCTCAATCGTTTGATG
GGGTTTACACAATACAATTGACAACATCACTTTCTTGTGGCGCTTACTATTTTAAGATAAACATTGTAC
GTTGGGGGTGAGGACTCCAACCACTAAGTTGCCAGATTATGTTTATATTGATGCTATTAG

***A. majus* 20o24-T3**

CNCNATCAGCTTGATACAGGCTGCCAAAAAAATTTCTTCGTGAATTACCCCAACTGGGACGCCAAGGTA
NTTGAAAGGTGTTGANCCAACNCTACAATTTAGCAGCTGCGCNAAAGAATTCATTTCTTCCNTTATATA
TTAGNATAGATATTACCCGCATGGGTACCGCGTGGCCCAAAAGCTAGTTTATGAATAAGCTAGTTTTGT
ATAAGCTTTACATTAATAAATTAGTCCAGTCTCCAACTATTCAATATTTTATCTAAGAACAACTGGGTC
TTTGTTAATCATATATCTAAGGAGGTGAAATAGCAAATTCTATTATACGTACATACATACACACACATA
TATTTATTTTGTAGATGTTCATTCTCTATCAGGTTTGGATGAGGTTTGGATGAGTATTCTTCGTTGGTT
GGGTT

***A. majus* 11h19-T7**

AACCAAGCTCGGTACCCGGGGATCCTCTAGAGTCGACCTGCAGGCATGCAAGCTTAGTAGCTGTTACAA
CAATATAAGGCCTTCAAATTGCTTTTATTATGCTAATCTAGAAAAAATCCCTTAAGCATTTCAAGATAG
ATTGATCTATTTTGATTTTCAGAAAATGTTCTTCATTTTGATGTCAGCTATACCTAAGCACTTTACTAA
CTTCTATGCTCATACCTCCTTTGTCTTACATGTTATCTGCATATGCATGGTTTGGAGCTGCAGCAGAAA
AATGACAAGTTCTTTTTCAATCTACTCTGATTACCACTGTGTTCCTTTAGTTGAATAATTTAGTACTTT
GAAGTCTTAAAATCACGTTGGAGAAATAGCTGGATAAATTTCAGAGGCACTCTCCATCCTGTTTAAGCA
GCCTTATANCATGGCAATGAGATTGCTGCTGCTGATAAANAAACTGTACTTTGTGTTGGGGACATTGGC
TATCACTGATATTCTTAGAAGGACTGGAAAATGAAAGTTTGCTTAAAATTAGNAAAGCCATCTNACCTG
GATTTTACTTTGTNAAGGTTTCACCTGCTAAACCTTTTCATTTCTCTTCTCTAATTNATNAATAGCTTG
TTATCGGTTGTTCCATGTGTAATCAGCCTTTTCCTAGNGGCAATGACCTGTATTTATTTGTCCCGGTGA
TCAGCTAGCAATAGGAAAAGGAACNGGGCGTCTCTTGGGTATGGAAACCAACATGTATCCCTCACTTCT
TTGGGGAGGGACAAAAACAAAAGATGAGNCCCNNTTGGCTAGTTTTGCCCT

***A. majus* 11h19-T3**

TAAACAAGAGCATGCAATTTTCGAACCCTAAGCATCAATATACATCAAAAGAAACTGCAGTATACACAT
AACAACAACAGAAAACTTATGCCATTGCCTCCCAATTTTTCAGTTTTCCATATCAATTTGGCCACTATT
ATACGCGATTGAACAGAACGGCGTCGTATTACTGAAGCTCGCGTTTTTTTTTTTTGGTAAATAATAAAC
ATTTGGTGTGATGACGTGAATATCGTTGCAGTATATAATAGTTCACCCTGTTTATACAGTAAAGCATCA
ACTTCTACTTTATTTTTTGGAATTGTTTAATACTTAATTTCATAATTATATATTTAGGATTTATTATAT
TACTTTCTTGATTTTCTCTTTCCCAAAAAA

***A. majus* 35m21-T7**

GTCCGGGGACCTCTGAGTCGACCTGCAGGCATGCAAGCTTAAAATGAATTTAAGTGTAACANATATAAA
ATAGGGAATTTTTTTTTGTCATTAAACATATGNCCGAGAATTTTACCAATTAAAAATAACACATGGTGA
AGACTGCTAGCTATATGTCAAAAAAAGTATATAAGAAAATAATTGCATTGCCGCGCTTTTCATCAATGA
TCATATGCATATTCGTTCAATTCATGCCTGTTGTTTAGTCATTATATATTCTCCTAGGATATTTAGGAT
TCCTAATTCATACGGGATTTTGAATGCATATCTATAATAACACAACCTCTTCCCCTATTTGTTTTCAGA
AATCATTCCATATTTTCCACAACTCAATTCACTTAATTTGTTTCTTTGAATTCGTTCTATTATTGATAA
AAAGATCGATGGATTATATTTGCAAGCCCTCTTCCCAAAAAGTAGTTCAAGAAACCAAATCTTGGTTAA
AGATTCCGAAATTGGAGCTTCGTAAAACCATCAAGAGTCGATATGAGGTGACTAAGTACTCACTGAAGA
TGAAGGAGAAAGCAGAGTGGCTCAGAGCTTCTCGGGATTACTCATTTTTATTCTCAGATGATTGTACTA
ATATTTCAGTTTCCGATCAACAAGTCTTGGGACGTAAGAGGAAATTCAAGAATCAGGATGAAGATGAGG
GGAAAATCGCAAAAGTTCCACGCGGTGTTACGGTTAAAGTTGTGCGACATGCAAAGGAAAGAACTGGAG
AGAGTGGTACTGAAGATGAGTTCAGCCTGGAATCTTGTATTCAAAAGCTATCAAAGTGCACAAGAAAAA
GATCGGTTCGGAATTGGAGAAGATCAACATGGATGGATCAGGTTTGGTTAACAAGTTATTTTAGTACTT
TTCCCCACTCAAAAAAAAAAAAAAAAAATGTATAGCCCCCGTTTGGTTAATTTGGG

***A. majus* 35m21-T3**

TGGATCTGATTAACAATTCATTGTGTAAGATTTTTACTTATTCAATGAGNCTACAGTTTGCTGGGGGCC
TCAGCAGNTAGAGGATTTTGGGAAGTGTTAGCATTTTGTGGANATCCGTGGTTCATAGGGATAGTAAAA
GAGCTGTCAATGGGTCAAGTTTTTATCAGAATCTGCTTAAACAATATATTGTGTATGGTATTTATTGGT
TAAATTTGAAATTTACTGATCCGATCTGTTCAAGATATGTACTTGATTATATTGGATTAGGTTTATACA
TAAATAATAGCATTTGTATAGATCATGTCAACTTGTTGGGCTGCATAATAACTCAACTACAACAGTTAA
TTTCATTTAATCGGCTAAATATATCTAGTGATTGCAGATGTGATGCAGTGTGAATATATCTATTTTTAA
ACTAGAGTGGAGAAAGATACACGCAAAAGTTAAAATACTTCACAGATCTTATTTTGATTTCTCCAAGCC
CTCTCAAAATTACCTCCCTCCCCGATCCCTTCAGTGCTGTATCAGTCTCTTATTCTTCACAGTTTAAGT
TAAACAATGTTTAATTGTATAAAATTAGCATCACTTATTATTTTCATATCAATGCATTTGGTAACTTTA

GTTAGTATGCTAAATAGAGCACTATTAGCCACCATATTTCCAAAAACATAATCTCAAAATAATTACACT
TACTTTTCACCATTACTTACTATCTTTTCATCAAGGGTGACACTTAGGTGGCTAATGACGAGACTCCTG
ACCCTTACTATGAGATACAAATTCTCTAAGTACCAAGAGGAAGGCGACTATCCAGTTCTACCTTAAGGT
TTTTGGTACTGAGGCTATCGATAAAATTACTTTTTTCAAAAACCCAAACCAACAAACAATCCNACTGAT
GAAATACATCTGCTAACTACATCCCTATTCAAATTNAGATGAATTCAGCCACAAATAATCCCTAC

***A. majus* 54i20-T7**

CTCTGAGTCGACCTGCAGGCATGCAAGCTTATTAGCTTCAAGGAATCAGGGTGTTGAGTTTGATTGCAA
AATCACAGATTGAATCTTAACCTAATTGATTGTTAAATCACACAGATGAACAACACAAAGTATAACAAC
AGATCAAAGTATTGATTAGCACAACAAACACACACGAAGTTACTTGGTTCAGCACAAGATCAGAGTGAT
CAAGTGCCTACATCCAAGGGAGAAAATGATCCTTTATTTCACAGGTTTTCAAAGAATTCTACAGAGTGT
TTCAATCTCACAACTCTCGTGTATCTCTCTATGAATTGTAAACAACAAAACTGACTATTTATATTTACA
GATTTGATACAAGAAAGTGAGTAAACCACTGCTCTAGTCTAACAGATTTTCCCGCTCAAAATCTGTTAA
GTTTCTAACTGAAACTAATCCAGCTCCAGCTCCAGCTCAGCAGCAGAGCTTTTATCCTCTGTATCCATT
GCACAGCATTGATGGTGAACCAACATGCTCCACAATTCTCCACCTTTGTTCAGCATCAACTATGCTACA
GATCATAACCTCCACCAGCCTTTTACAAAGACTTCACCAGCCTATTCTCACAGGCTCCACCAGCCTATT
CTCAGGCTATCTTTCAGTCAAGTTTATCAACTCCAGGCAGTACTGAAACTTGCTGCTTGGTAACACTTT
AGTGATCATATCTGAAGGGTTATGATCAGTTGATATCTTCTTTACATTCAGAGCTCCTTTAGTTATCAC
TTCTCTAACAAAATGAAGCTTGATATCAACATGGTTTTGTCCTTTTCATGGTACATTTGATTTTTGGAC
AAATGAATTGCACTTTGG

***A. majus* 54i20-T3**

CGGCTCGACGTGGTACACGATCAGCTCCCTGTGATGCTGTCGGAGATCCTACCAGATCGGCCCGATGGA
GACCATGGGATATACGAGATCCTGCAGGTGTGCCAGTCGATCCTCCTGCAGGTGCCCCAACGGATCCTC
CAGCGGACCCACCAGGAGATGGAACACCTGAATGATGTTGCCGAGGGGCGGACACCGTCCCAAAAGGTG
CGCTGGAAGGCGGACTGGGTACAATGCCCGGTCGCAATGGAGGTGTCGACCCGGTCGGTGTCCGGTAGC
GCGAGGTGGCCCCAGATGACGCAGAACCTGGCTGGTCATCACTAGGCGATGACCGAGTACGAGGTGATG
CTCCTGGGACAGCCTGTCCTGCTGCATCGCTCGGAATATGTCCATCTGCCGCACTAGGATTAGTAGACT
GTCGTCTACGAGTCGTGATCTTCAATTTCCTCTTCGCTCCAGCTGAACCTGACTCACGCGGTGGCTGCT
GATCTGCCATCTATAACATTCACATACAATTAATCAATTACAGTAGCTAGCAATAGATTCAATAATTAT
CAAAACAACAACAATAATTGTCTCTCCAATTTTATTACAACTAAAACTCGCTCTAAAACACCGAACAAA
CCAACTCAAAATGCAAAACACCATATTTAGACAATAAAATTTCGATTGCACGTACTAATTAAATAACAT
CATAAACTCAATATAAACGATAAATTACTAAACAAACAACATACGCGACGTATTATCCATTTAATTACA
TAGCATTAATGTCGTAACATGCAACATAAACAAATAATTACATAAATAACTGATAACACTCAATTAAAA
CGATAATATACGCATACAACATCAACACGATTTAACGACTA

***A. majus* 89k05-T7**

TGCAGGCATGCAAGCTTTAGAGAAAACTCAGACATGGACACTCACTAATCTACCACAAGGACATCATTG
TATAGGATGTAAATGGGTATATAAAATTAAACATAAAGCTGATGGAAGTATAGAGAGATACAAAGCAAG
GTTAGTTGCGAAGGGGTATACTCAAGAAGAAGGGTTAGATTACTTTGATACATTCTCTCTTGTTGCCAA
ACTTACCACAGTTAGAGTACTCTTATCAATAGCTGCTTCTAAAGGCTGGTTCTTACATCAATTGGATGT
TAATAATGCCTTTTTACATGGTGATTTAGAGGAGGAAATTTACATGACACCTCCTCCAGGTTATTGTCT
TCCTAATGATAAACGTGTTTGTTTATTAAAAAAGAGCCTTTATGGTTTAAAACAGGCTTCTAGACAATA
GAACAATAAGTTAACAACATGTTTAAAAGCTTGTGGTTATGTTCAAAGTGTTTCAGATAGTTCTCTGTT
TATCAAAAGTTCTTCTATCTCTTTTACCACCTTATGTGTTTATGTTGACGATATCATCTTAACTGGTAA
TGATATGAAAGAGATCATCAACATCAAACAACATCTTGACAATGAATTCACTATTAAGGATTTAGGGAC
CTTGAAATATATACTAGGCATTGAAGTAGCTAGAAGCAAACAAGGCATTAGTCTCTGCCAAAGAAAGTA
CACTCTTGATATTCTTAAAGAAGCTGGATATCTTGCTTGCAAACCTAGCTCTACTCCAATGGATGTATC
CCCTAAATCTAAACCTAATGATAATGACTTACTTCCTGATCCTTCTATTTACAGGAGACTTGTGGGAAG
ATTGCTTTACCTTACTATAACAAGACCAGATATCAGGTTTAGCATTCAATCTCTATCTCAAAACATGTC
TCAACCTACAAATAAACATCTTGAAGCAGCTTCACAGGGTTATCAGATATC

***A. majus* 89k05-T3**

CAAAGAACTTTGTCATTCGCTGCAAAATAGGGTTCCCCGTTTCAATAATGAAGCCAAATACGAGGTGTT
GCTGACAAGCATGAGGTTGGCTAAGGAAGTAAGGATGCTTCGGGCTCATTTCCACAGTGACTCTCAGCT
GGTGGTGCAGCAGGTTAACGGGGCATACGAAGCCCGCGACGAGCGCATGAAAGATTACTTAGCCGCGGT
GCGACGATCGCAGGATGGATTCAAGGAGTTCACCCTCACACAGATTCCCCGAGCGAAGAACGAAGAGGT
TGATGCTCTATCCAGGGTAGCAGCCGGGATGCACTCCATAGGAACTCGACAGGTAACCTTCTTGATTGT
CAATTCTTCAGAAAATGATAATATAGCACTAGAAGCACTCCATATTGACTCTGGAAGCAAAGGTGGATG
GATGTCAAAAATCGTTGCTTATCTGCAAAATCCTGAGAAATACGAAGATGATGGAGTCTCATGAAGCTT
GGGTAAAAAGGCTACCCATTTTTTTCTCAATAACGGCGTTCTATACAAGAAAGGATTTTCCCAACCATA
CCTACGCTGCCTGGATGAAGATGAGGCAAAAGAAGTCCTTAGAGAAATTCATGAGGGAAGTTGCGGAAA
TCATTCTGGAGGAAGGAGCCTTGCCCAAAAGGTACTTAGACAAGGATACTTCTGGCCCACTATGCAGAG
CGATGCGAAGGCCTTCTCACAACAGTGTGATCAGTGCCAAAGATATGCCGCTATGTCCCATCTCCCTGC
GACCGCTTGGGAACCGAATCATGTTCCTTACCCCTTTTGACAAATGGGGGATCGATATAGTGGGGCTCT
TAATGCAGGATTGAGGGCAAAAAGGTATCTTCTGGTCGCCGTGGAATACTTCACGA

***A.majus* 18d03-T7**

CGNCCGGGGACTCTGAGTCGACCTGCAGGCATGCAAGCTTCTAAGGATCCTCATTGGATCTCTGCTATG
GAAAAGGAGATTGATGCACTTGAGAGCAATCATACTTGGGATTTAGTTCCCCTGCCTAATGGTAAACAT
CATGTTGGCTCTAAATGGGTATATAAGTTGAAGTTGAACCCTGATGGGAGTATAGAGCGTTACAAGGCG

CGTCTGGTAGCTAAAGGCTACACACAAGTGTTTGGGTTGGATTATTCTGAGAGTTTCTCTCCAGTTGTG
AAGTTGGTAACAGTCAGGATCCTCTTAGCTGTGGCTATTGCATCTCATTGGTGTCTTCATCAATTGGAC
ATCAATAATGCTTTTTTACATGGTTTTCTCGATGAGGAGATATACCTTACACCTCCTGAAGGTTACACA
AAAGCTTTACCAGGCTATTGTTGCAAGTTAAACAAGTCCCTTTATGGTTTAAAGCAAGCATCCCGCCAA
TGGAATCAGGAATTCACTAAGTGTCTCATTAATTTGGGCTTCTCTCAGTCTGCCTTCGACCATTGCTTG
TTTACCAGAGGTTCTAATTCCTCTTTCCTGGCCTTGTTGGTTTATGTTGATGATGTGCTGATAACAGGA
CCTTCTCTTGCTCAAATTACTGCTGTTAAAAATGAGCTTCATAAGGCTTTCACTATCAAGGATCTTGGG
GATGCCAAGTACTTCCTTGGCTTAGAGATCATAAGACATCTTCAAGTATGTTTGTCAATCAAAGGAAAT
ACATACTAGATATACTCAGTGATGCAGGGAATTCTGGGTGCTAAATCATTGGATGTTCCATGTCAAAAA
TCATTCAAATTGGTCACTGAAGGTACTGCCTATCCTAAGCCTGATCAATACAGGCGTCTTTGGGGGGGA
GATGGCTTT

***A.majus* 18d03-T3**

GTTATATGATATGGTGCAGAAATTACGACCAGTGACATTCTACGACCTATAGGTTACGATGCCGGCCCA
AGGAAGTGTACGACCCAAGATTCGAGATCAAAGGAGAAACGCCGAAGGGAATGTCAAAGTAGCACTTGA
TAACGACTACGACGCACTAAACGACGCCAACATCGTAGACGGAGAATACTAAGTTAGACTGACACTTGG
TAGACAGGCATTAATAGAGGCTCAGTGCCTCGGAGTCAACACCTGTCGGCCACTAGGTGTCAGGCGTTA
ATATACGACCCTCGATGCAGGCAGCTAGTAAATGCAGCCTTAATGATCTCTGTCATTCAGGGCATTTAC
AAACCCGAAGTCAACGAATCATGCGCGACAACCGATTCGACACTTGTCATCGGAGGACCGCCACGTGCT
TAACTTCGACGACCTATAAATAACACGGTCGATCAAACCGAATCACCAGGCAAAAACAATGAGTAAAAT
ACCAGATTTAGAGAGAGAAAAACCCACCACTCACTTGGTTCTAATCGAGGTACGATTCGAGGAGAACAC
TTGAATTCGGGAATAAAGAGTATTCCATCTTGTATCCTCACCTAGATAAACTGATAACACCTTCGTAGC
GTCGAATCCAAAATCACCTTTATCAGTTTGGCGCCGTCTATGGGAACGACTCTTAATATTCTCTGAGAA
AGCCAAGATAGAAGTGAGTGTGAGAGACATAAAGTTCGGATTTAGCGCATAAAATGAATGGTGATGGGG
CTGAAACAAGTAGGAGAAGCCCGCCATTGGCAAGCAGCGGATCTAGTGGGCGCCCTCACCCTGCCGACT
TCATCTATATTTCGAG

***A.majus* 22l05-T7**

CGCCGGTCCGGGGANCTCTGAGTCGACCTGCAGGCATGCAAGCTTGTGTGGAATAATTTGGAAGATCCT
TTGAGTCAAACTGTAAAACAAGTTCATCTTGTCTTTGATCTATATTTAGACATTCAGTCTAGCCTAAAA
TGGGCGAAGGGTAGTGAAAACATCAAATTGTTCTTGAGGAAGGTTGCTTCTCATCTCCTTCGTCTTGGA
CCACGTTGCAAGGGAAGATATGTTCCTTTAGCTTCCCTTACAAGGAGGTTGGGGGCTAAGACAATTCTA
GAGATGAATCCGGACATGTTGTTTGAATCAACTAAGGCATACATTGATGATGATGTGTGTTGTGCTGTT
ACAACATTCCTCAAATGTTTCCTTGAGTGCTTACGAGATGAATATTGGAGTAGCGATGGTGTGGACGGT
GGATATAAACAATATAGAGGTCATTGTTTGCAACCATTTCTGCTTGGATTGGCTTCTGGAGTGGCCAAG
TTACGCTCAAACTTGAATACTTATGCATTACCGGTNCTACTTGAGGTGGATGTGGAC

***A.majus* 22l05-T3**

CTACCAGCTGAGGGGCTGCTGCTTATTTCGTAATTGCCATAATTACGATCTCGAATCTACTCGAGTTAG
ACATTTGCAATCGACTTTTGTTTGATTTAATTGATCAAAAGTTTAAGACGCATGGNAAGTACGTTAGAA
AAATTGGTAGAATATACAAGTTAACACTTAACACCTCAACACACTCAAAAAAGTGGAGGATGAGTTTCT
ACAATTCAACTCTAATTAACTTTCAAAATGAATAGATTACATAAGTATTTATAGAATTTTCCTAACATT
AACCTAACTTTGTTAATGTACCATAACTTAATTTGCATATATTCACATTTCGTAGTATATGAACATAAC
AAAAGATCTCCAGTCCCACTTAACTACACACTGTCCATGTTTTAAGATTAAGGGCACGTGTCATCCACC
AACTCTGGCCGCTAAGATCAAAGACACATGTCATCATCATAAAGAATGAGACAAAAAGTTGGACAAATA
TTCTTACCGCATGAACACATGCAAATGACATAATGATGCTCATTGTAAATCTTGTATGTACGACATTAT
TACAAACACTCCTAACAATTCATGCACTTTATTAAGACAACGAACCTAGTTTATTATTGGACCTACACG
TGAGGAAGCGTGTTGAAATTTATTCTACGTTGCTCGTAAATGAATTTGAGCTTCTATATATTAATAATA
TCTCATTCTTATAAGAACTTGAGAAATATGAGTGGTTCTCTACATAATAGATTTTTTTTTCTCTTTGAC
GCCAATTTTTCGTTCGGCATTACTGCATGAACATGACATCCCATTTGACAAAAATCCATTGCAAGTGTA
TAAGCCAATATTGCAAATATATAAGAGTAAGTACAAGATGATCACATTTCTCAAACAAACTCCCAGAAG
CAAAACATTATTACTCGATCGCNCGCGGTGAATTAACCTCNAGGGGGCAAAAA

***A.majus* 30m23-T3**

CTACCGCTGAAGTAAAACCTCGTTACTTCCTTTCTTGATCGTGGCCAGAGGAGTGCACCATTTTCCTCC
CGAATCCTATNCACCAAATTAACAATGTAGTAATTNATATAAAGTAACGAAATATATGTATAAAAAGAA
AAAAAAATGATTGATTATGTTATGTAAGAAAGTTTACCAGTAGTGAACAGGAGGATGAAAATTGACAGA
ATAAGCACCATGCTAAAACAAGATCGACCCATTTTAAGTCCTATATGAAAATAGGTAAAGTGAATGAGA
AAGAAAAGTACTACAGTCTCAGATCCGGTGAATAGATTTGATACGATGGTTTTAACGATAGAGACGTGT
GCATTGTGGACTATTGAGACTATTATATACAAGTTTAATCATATTTTTTCTAAAAAATTTGTAAAGTCA
AACCGTTTATGTACTTTGATTCGTAAAATATAGAGTTATTTTTAGCAAGTTATGAAATATATTAATGCA
GTTTTCTACTGGTGAGCTGGTCGAGGAACTTGGTGAGATGTTTCTGAATAATATTTTTATTAAGTTTAT
TAGTTTCTTGGATATTGTAACCGTCTTACTATATTTTGGGGTAATAGCTAGGAGGAGGAGAAGATGTAG
GATCTTG

6.2 AFLP primers used for fine mapping of the *FIS* locus

Name	Sequence (5'-3')
(PREAMPLIFICATION)	
ECORI-01	GACTGCGTACCAATTc
PSTI-01	GACTGCGTACATGCAg
MSEI-01	GATGAGTCCTGAGTAa
(SELECTIVE AMPLIFICATION)	EcoRI-01+3
ECORI-31	GACTGCGTACCAATTCaaa
ECORI-33	GACTGCGTACCAATTCaag
ECORI-34	GACTGCGTACCAATTCaat
ECORI-35	GACTGCGTACCAATTCaca
ECORI-36	GACTGCGTACCAATTCacc
ECORI-37	GACTGCGTACCAATTCacg
ECORI-38	GACTGCGTACCAATTCact
ECORI-39	GACTGCGTACCAATTCaga
ECORI-40	GACTGCGTACCAATTCagc
	PstI-01+2
PSTI-11	GACTGCGTACATGCAGaa
PSTI-12	GACTGCGTACATGCAGac
PSTI-13	GACTGCGTACATGCAGga
PSTI-14	GACTGCGTACATGCAGgt
PSTI-15	GACTGCGTACATGCAGgg
PSTI-16	GACTGCGTACATGCAGag
PSTI-17	GACTGCGTACATGCAGat
PSTI-18	GACTGCGTACATGCAGct
PSTI-19	GACTGCGTACATGCAGta
PSTI-20	GACTGCGTACATGCAGca
PSTI-21	GACTGCGTACATGCAGcc
	MseI-01+3
MSEI-31	GATGAGTCCTGAGTAAaaa
MSEI-32	GATGAGTCCTGAGTAAaac
MSEI-33	GATGAGTCCTGAGTAAaag
MSEI-34	GATGAGTCCTGAGTAAaat

continue →

Name	Sequence (5'-3')
MseI-35	GATGAGTCCTGAGTAAaca
MseI-36	GATGAGTCCTGAGTAAacc
MseI-37	GATGAGTCCTGAGTAAacg
MseI-38	GATGAGTCCTGAGTAAact
MseI-39	GATGAGTCCTGAGTAAaga
MseI-40	GATGAGTCCTGAGTAAagc

6.3 Primers used for map-based cloning

Name	Sequence (5'-3')
fw001	CGTACCAATTCAAGCAAAGTC
rev001	CACTGGTGGAAACCAATACC
004fw3	CAGGCAACCGGAATAGCAATTCCCATT
004rev3	CCATCAATTTTCTCTGAATACTTATAGCTC
005fw3	AGGTAAGGATAATTACATTGACACCTCC
005rev3	CGAGTATCCATAATGTGAAGAGGGATGC
996*	GCAATGGCTTCTTCTGCAGCTATCAAGC
997*	CGTATGCTGGGATTTGATACACAGTGAAC
0008	GCACGAGGAAATGCACAACTTTGC
0010	AACTCGTTCCGAATTGACCCTTTGCAGG
0019	AAGCAGTCTCCAATCTTCGAACTGTGACGG
0020	TAACACCGTCTGCTATAAGTTTGCCGCC
0021	CAGATAAACCCTGAAAGCACAC
0022	TTGCTAGGAGAAGCCACTGGAGAAGC
0025	ACCATTACCTGAGCAATCTTGATGGCCACG
0026	CTTCCACGTGTCTATGTAATGAGG
0040	TCATCTATGACATGCAACACGGAGG
0041	TTACCACGTATGCAAAGTTGGGGC
0044	TGAGAGGTAGCAAAAGTGGACAACAGTCC
0045	AGGCACCACTTCATTAGAGAATTGGTCG
0046	TGGGCCATGTTAGAAACTTTGGGC
0047	AATTAATGTGCACGGGGCTCC
0049*	TGGATGGCTCTCTCAAGAC
0050*	GTTCCCATCTTGACCAAG
0053	GATATTACCCGCATGGGTACC
0054	CCAAACCTGATAGAGAATGAAC
0059	ACCTTATGCCATTGCCTCCC
0060	TTCACGTCATCACACC
0072*	ATAATTGCATTGCCGCGC
0073*	TCCCAAGACTTGTTGATCGG
0087	AACTGTGAACTGAGAGGAGC

continue ▶

Name	Sequence (5'-3')
0088	CTAATTAAGCACAGCGGAGG
0123	AGCAAGGTTAGTTGCGAAGG
0124	AGACAATAACCTGGAGGAGG
0151*	TTAGTTCCCCTGCCTAATGG
0152*	TTGAGCAAGAGAAGGTCCTG
0171	ACTTTAGCTAGAGACCCTGC
0172	ACTCCGTCTAGTGTGTTGAG
0173	ATTAGGATCAGAGCTCGGAG
0174	GATCCATAATTGTTCGGGCG
0175	CGATCTCGAATCTACTCGAG
0176	TCATGCAGTAATGCCGAACG
0186	TACCAGTAGTGAACAGGAGG
0187	TCCTCCTCCTAGCTATTACC
0204	CGTCAAGTGGTTATGAAGACC
0206	CTCACTCACCAAACACACGG
5172	TGTTGCACGAACATGTTTC
5173	CCTCTAGAATCCCTTTGG
5174	GCATTGTTTACTAGTGCTTTTAGGC
5175	GTCATATATCCCCTTCATC
5176	GCCTCATATCTACCCTGAC
5177	TGTTGCACGAACATGTTTC

* indicate primer used for physical mapping of the genomic contig to *FIS*.

6.4 Isolation of BAC clones belonging to the *FIS* contig

Probe	Primer pair	BAC clones	Insert size (kb)	Proximal end
53b14-T3	005fw3/005rev3	40g15	50	
		74b15	55	74b15-T7
74b15-T7	0028/0029	n/a	n/a	n/a
53b14-T7	004fw3/rev3	12h18	95	12h18-T7
		43o09	n/a	
018_6_05_m04 (in 12h18)	0008/0010	15l03	n/a	
		31f16	n/a	
		45n02	n/a	
		57f23	n/a	
		84h10	n/a	
		89g16	95	89g16-T7
89g16-T7	0025/0026	15l03	n/a	
		57f23	n/a	
		61n17	110	61n17-T7
61n17-T7	0040/0041	20o24	95	20o24-T3
		62o18		
		64h08		
		66c12	95	
20o24-T3	0053/0054	11h19	110	11h19-T3
11h19-T3	0059/0060	16p12	90	
		35m21	80	35m21-T7
35m21-T7	0072/0073+	01h06	90	
		18n07	100	
		35a08	110	
		47f11	80	
		51m18	80	
		54i20	120	54i20-T3
		61n11	80	
		71g08	70	
		74g21	80	
54i20-T3	0087/0088	54j16		
		89k05	70	89k05-T7
89k05-T7	0123/0124	11i24	80	

continue →

Probe	Primer pair	BAC clones	Insert size (kb)	Proximal end
		18d03	110	18d03-T7
18d03-T7	0132/0133	58d17*	100	
		22l05*	80	22l05-T3
22l05-T3	0175/0176	30m23*	100	30m23-T3
		35h17*	n/a	
		90g22*	n/a	
30m23-T3	0186/0187	88h23*	95	88h23-T7
88h23-T7	5176/5177	62j11*	80	62j11-T7
62j11-T7	5174/5175	n/a	n/a	n/a

+: primer pair used to map the BAC end to the FIS locus.
* :BAC clones falling into the *fis*-1 deletion.

6.5 ESTs contained by the isolated BAC clones

BAC clones	NCBI annotation	BLAST homology
74b15	018_1_04_p04	unknown function
	018_2_10_o09	phospholipid acyltransferase family
	018_2_10_o14	senescence-associated protein
40g15	018_1_04_p04	unknown function
	018_2_10_o09	Phospholipids acyltransferase family
	018_2_10_o14	senescence-associated protein
53b14	018_1_04_p04	unknown function
	018_2_10_o09	phospholipid acyltransferase family
	018_2_10_o14	senescence-associated protein
	018_3_06_o16	WD-40 repeat family protein*
12h18	018_6_05_m04	endonuclease/exonuclease/phosphatase
	018_3_07_n12	unknown function
89g16	018_6_05_m04	endonuclease/exonuclease/phosphatase
	018_3_07_n12	unknown function
	018_5_03_c17	oxidoreductase
	018_6_04_f07	unknown function
	018_5_03_c17	oxidoreductase
61n17	018_6_04_f07	unknown function
	018_6_11_c10	phosphotransfer family protein
	018_6_02_j10	glycosyltransferase family protein*
20o24	018_6_11_c10	phosphotransfer family protein
	018_6_02_j10	glycosyltransferase family protein
11h19	018_3_08_l06	Acid phosphatase
35m21	018_1_01_n06	NADH-ubiquinone oxidoreductase
	018_3_07_f07	unknown function
	018_3_06_o05	cyclin family protein
	018_3_07_b19	BolA-like family protein
54i20	018_3_02_e12	homeobox-leucine zipper protein
	018_5_01_e12	polyprotein
89k05	018_3_02_e12	homeobox-leucine zipper protein
	018_5_01_e12	polyprotein
22l05	018_1_09_o04	calcineurin-like phosphoesterase * +
30m23	018_6_02_n16	myb family transcription factor +

continue →

BAC clones	NCBI annotation	BLAST homology
88h23	018_1_12_n05	zinc finger homeobox family protein +
	018_3_06_l20	stearoyl-ACP desaturase +
	018_3_12_g11	MIP family protein +
	018_3_11_e22	FRIGIDA +
	018_5_08_j13	F-box family protein +

* ESTs used to develop markers (see text).
+ ESTs present in the *fis*-1 deletion

6.6 Oligonucleotides used for other applications

Application	Name	Sequence (5'-3')	Gene Amplified
Genome Walker	MIBUS474	ATGCAGCCAAGGATGACTTGCCG	miR169 core
5' RACE miRFIS	0330	CGTCGGCAAGTCATCCTTGGCTATACCTCG	Pri-miRFIS
RT-PCR miRFIS	0555	CCCAATTGGCTTCTCCCTTTTGAC	Pri-miRFIS
	0664	GCCAAATCAATTTGCACTTCAGGTGG	RAN3
	0665	CGAATCAACAAACGGTGGATTACCC	RAN3
AmYAs cloning	0221	CCTCGGATGTTCTCGAATGG	AmYA3 3´ seq.
	0222	ACGTGCTAAAGCAGAGCTGG	AmYA4 3´ seq.
	0223	TTTTGAGATCGGATACGGCC	AmYA1 3´ seq.
	0224	TTTATTTCCCCGCATCATTGCAGGGG	AmYA4 5´RACE
	0225	AATTTCTCCAAAGAACCCGGCC	AmYA4 5´seq.
	0226	TTGTGGTGCAGATATGGCTTCC	AmYA1 5´RACE
	0227	ATTCTCCATCTCTTTCTCCGCGC	AmYA1 5´seq.
	0228	AAAAAACAAATGAGGATGCACCGGTGCCCC	AmYA3 5´RACE
	0229	CATTATTCCCCCATACTGTGG	AmYA3 5´seq.
	NFYA1F	CTAGCTCTGTGGCACATTCAACTCC	At1g17590
	NFYA1R	GGAACGTACACATTACCAGATTCCACC	At1g17590
	NFYA2F	CAGCTACTCATTCCACTTTGCCATACC	At1g72830
	NFYA2R	CATGAACACAGCACCAAGATCCACTGTC	At1g72830
	NFYA3F	GCAACTTCTTGGGATAACTCTGTCTTC	At3g14020
	NFYA3R	GCTCTCTCACATGAGGACTGAGACATGG	At3g14020
	NFYA4F	CATCTTGGGGAAACTCAATGCCTAC	At1g54160
	NFYA4R	GAGGCATGGTGTGTTGACGGATAACC	At1g54160
	NFYA5F	CAGACTTGTAACTAGGACAGTCTTCTC	At2g34720
	NFYA5R	GTGCAGAAACCTTCCTCAAGATCTACC	At2g34720
	NFYA6F	CTGCTTCGGACATTAAGCTCATGAGTC	At1g30500
	NFYA6R	CTTGTAGGACCTTCTCAAGATGTACC	At1g30500
	NFYA7F	CACAGACTTCTTGGTGGACTGCTTTTGG	At3g05690

continue →

Q-PCR	NFYA7R	GAAATTGCAGCAGCCATTATCCATTGC	At3g05690
	MIBUS696	GTTTGTTGTGGGTGTCAATGAGA	GDPH (E: 94.9%)
	MIBUS697	TGCTGCTAGGAATGATGTTGAATG	GDPH
	MIBUS698	CTTTATGCCAGTGGTCGTAC	Actin (E: 94.3%)
	MIBUS699	GTGAGGTCACGACCAGCAAG	Actin
	MIBUS702	CCAACGCAGCACAAGAACCTC	AmYA2 (E: 97.4%)
	MIBUS703	CCAAGGATGATCTGCCAGTTACC	AmYA2
	MIBUS704	CAGAAAGCAAAGCAACCTTCAG	AmYA4 (E: 95.4%)
	MIBUS705	AACACAGCCAAGAATGAATTGC	AmYA4
	1455	GCTTGGTGAGGGTGTTAGCAATATGG	PLE (E: 93.6%)
	1456	CATCGTACGACTGTGAAGTCATCG	PLE
	1457	CGTTGAGCATGAGTACTGATCAAGG	AmYA1 (E: 99.8%)
	1458	GTCTTTTCGTTGCTGTCGTCATTTGG	AmYA1
	1459	CCTCAACACAATCTTACTCATCTGC	AmYA3 (E: 95.6%)
	1460	GCAAGTTCCAATTGTCCTCACTCG	AmYA3
	1463	GAGGAAGTTCTGGCTATGAGC	FAR (E: 83.6%)
	1464	CATTTGCTCGAATCTTGGCTACTCC	FAR
	1465	CCTATGAGGCACAACCTTACCCAG	AmYA6 (E: 95.8%)
	1466	GCTTTACCTTGCGAGACTTGGC	AmYA6
	1467	GAGCGTAACATCCAAATCACAAGG	AmYA5 (E: 96.2%)
	1468	GCAAATGGCAGAGAGGTTCCAG	AmYA5
Nicotiana infiltration	GW1	GGGGACAAGTTTGTACAAAAAAGCAGGCTAACTGGCAGATCATCCTTGGCTATGAAATTTACTGCTTTTCC	AmYa2 wt 3´UTR *(forward)*
	GW2	GGGGACCACTTTGTACAAGAAAGCTGGGTTGTTCAAGTGCAGGTTCAAACCACAACAAATTGCC	AmYa2 3´UTR *(reverse)*
	GW3	GGGGACAAGTTTGTACAAAAAAGCAGGCTAACTGGCAG*tatgcg*CTTGGCTATGAAATTTACTGCTTTTCC	AmYA2 mt 3´UTR

7. LITERATURE

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