Molecular analysis of petal development by X-ChIP and two-hybrid technology

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„Eppur si muove…“

Galileo Galilei (1564-1642)

Father of the scientific method

Abbreviations

3-AT	3-amino-1,2,4-triazole
A. tumefaciens	Agrobacterium tumefaciens
AGAMOUS	AG
AP1	APETALA1
AP2	APETALA2
AP3	APETALA3
b-gal	b-galactosidase
bp	base pair
CaMV	Cauliflower mosaic virus
cDNA	complementary deoxyribonucleic
CHS	CHALCONE SYNTHASE
CIN	CINCINNATA
CYC	CYCLOIDEA
DEF	DEFICIENS
DICH	DICHOTOMA
E. coli	Escherichia coli
EDTA	ethylene diamine tetraacetic acid
FLO	FLORICAULA
GLO	GLOBOSA
GR	Glucocorticoid Receptor
GRX	GLUTAREDOXIN
GSH	glutathione
HEPES	4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid
IPed DNA	Immunopreipitated DNA
kbp	kilobasepair
LFY	LEAFY
MCM1	MINICHROMOSOME MAINTENANCE1
MEF2	myocyte enhancer factor 2
m-pre	mutant immunoprecipitated with pre-immune serum
mRNA	messenger ribonucleic acid
MS	Murashige and Skoog
m-se	mutant immunoprecipitated with serum
NAOAC	sodium acetate
NAP	NAC-LIKE, ACTIVATED BY AP3/PI
NH4OAC	ammonium acetate
nsf	number of specifically immunoprecipitated species
PAN	PERIANTHIA
PcG	Polycomb Group
PEG	polyethyleneglycol

PI	PISTILLATA
PLE	PLENA
PTL	PETAL LOSS
PVP	Polyvinylpyrrolidone
RBE	RABBIT EARS
SAM	Shoot Apical Meristem
SDS	sodium dodecyl sulfate
SEP1,2,3	SEPALLATA 1, 2, 3
SQ-PCR	semi quantitative-polymerase chain reaction
SQUA	SQUAMOSA
SRF	serum response factor
TRX	THIOREDOXIN
wt-pre	wild type immunoprecipitated with pre-immune serum
wt-se	wild type immunoprecipitated with serum
X-ChIP	Crosslinked Chromatin Immunoprecipitation

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1. INTRODUCTION

1.1 Regulation of floral organogenesis

1.1.1 Determination of the flower meristem

In flowering plants, the transition from vegetative to reproductive growth is a critical developmental process that involves the shoot apex and is marked by a number of changes at the molecular, physiological and morphological level. The switch from vegetative to reproductive development leads to the production of flowers instead of leaves or shoots (Steeves and Sussex, 1989).

In *Arabidopsis* and *Antirrhinum*, instead of the vegetative meristem, an inflorescence meristem is formed, that is composed of undifferentiated cells that proliferate indeterminately, allowing the plant to generate new flowers throughout the life span.

These undifferentiated cells are moved to the flanks of the meristem by continued cell division, and enter a specific developmental pathway leading eventually to the floral meristem formation.

Some of the genes involved in the transformation of these undifferentiated cells into the floral meristem were isolated: *LEAFY* (*LFY*) (Weigel et al., 1992) and *APETALA1* (*AP1*) (Mandel et al., 1992) in *Arabidopsis* and *FLORICAULA* (*FLO*) (Carpenter and Coen, 1990) in *Antirrhinum*.

LFY and *AP1* play a central role in turning the indeterminated inflorescence meristem into a determinated floral meristem; in fact flowers of *lfy ap1* double mutants are partially transformed into inflorescence shoots. Furthermore, *AP1* and *LFY* play a

crucial role in the activation of flower homeotic genes that control floral organogenesis (Lamb et al*.,* 2002).

1.1.2 The control of floral organogenesis in *Arabidopsis* and *Antirrhinum*

The flowers of *Arabidopsis* and *Antirrhinum*, like the typical flowers of eu-dicotyledonous plants, are composed of four different types of organs. Each organ type is arranged in a concentric ring or whorl, numbered one to four from the outermost to the innermost. In the first and second whorls sepals and petals develop, respectively. The reproductive organs, stamens and carpels, develop in whorl 3 and 4.

After the floral meristem is determined, organ identity genes are activated to govern organogenesis, until organ maturity is achieved. Organ identity genes, also known as floral homeotic genes, determine the development of organs in the four whorls. A mutation in these genes causes the conversion of one organ type into another organ type (reviewed by Jack 2004).

Genetic studies in *Antirrhinum* and *Arabidopsis* using floral homeotic mutants led to a simple model (Coen and Meyerowitz, 1991) proposing four classes of genes named A, B, C and E to function in adjacent whorls of a flower.

The class A genes, that include *AP1* and *APETALA2* (*AP2*), exert their homeotic function in the two outermost whorls. Loss of the A function in *Arabidopsis* causes transformation of first whorl sepals into carpelloid leaves in weak A-function mutants, and second whorl petals become stamenoid (Gustafson-Brown et al., 1994).

Genes from class B, *APETALA3* (*AP3*) and *PISTILLATA* (*PI*) in *Arabidopsis* and *DEFICIENS* (*DEF*) and *GLOBOSA* (*GLO*) in *Antirrhinum*, are expressed in the second and third whorl. When class B genes are mutated, sepals and carpels replace petals and stamens, respectively (Sommer et al., 1990; Jack et al., 1992; Tröbner et al., 1992; Goto and Meyerowitz, 1994).

The class C is represented by *AGAMOUS* (*AG*) in *Arabidopsis* and *PLENA* (*PLE*) in *Antirrhinum*. In C function mutants, petals develop in whorl 3 instead of stamens, and new flowers are formed inside whorl 3 in *Arabidopsis* and inside whorl 4 in *Antirrhinum.* This observation revealed that class C genes exert also a role in

meristem determination, other than in organogenesis (Yanofsky et al., 1990; Bradley et al., 1993; Davies et al., 1999).

The observation of the A, B and C class homeotic mutants led to the development of the ABC model that describes their genetic interaction. Class A genes are able to guide the development of organ primordia in the first whorl, giving rise to sepals, and genetically interact with class B genes to determine the organogenesis of petals in the second whorl. In the third whorl, B and C function genes determine stamen development, and the expression of the C function genes results in carpel development in the innermost whorl (Figure 1).

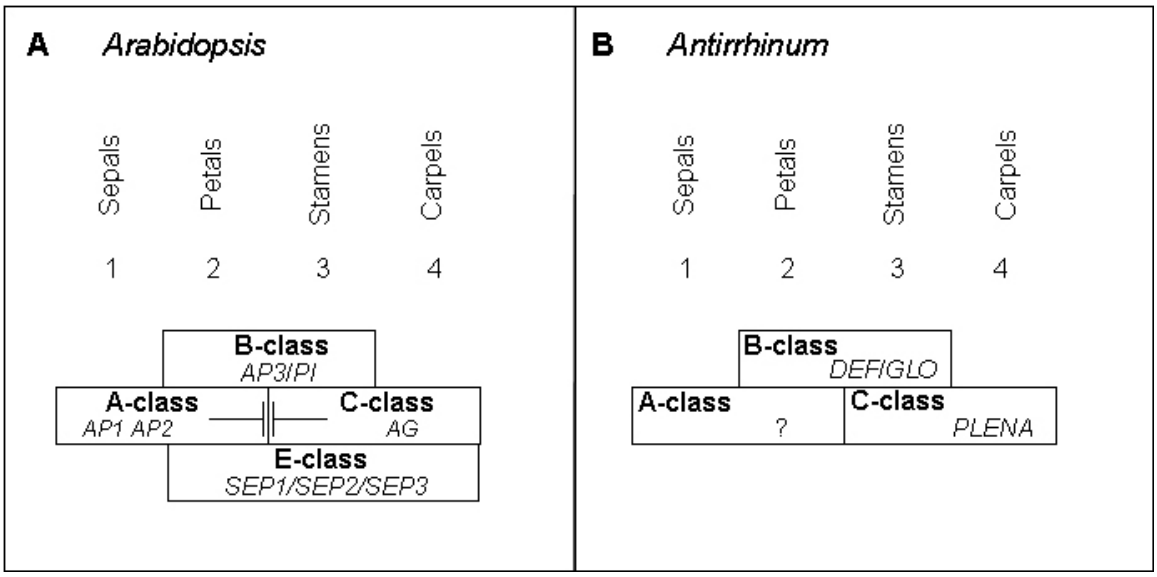


Figure 1. The ABCE model
A) The ABCE model in *Arabidopsis*: in the first whorl sepals are determined by A class genes, in the second whorl the A, B and E function determines the development of petals. B, C and E class genes together promote the development of stamens in the third whorl. In the fourth whorl the class C and E lead to the development of carpels. Homeotic genes belonging to the different classes are indicated.
B) The ABC model for *Antirrhinum*. Class B and C genes are indicated. Class A function genes have not been isolated yet.

More recently the class E genes were isolated in *Arabidopsis*: *SEPALLATA1, SEPALLATA2, SEPALLATA3* (*SEP1*, *SEP2*, *SEP3*) (Pelaz et al., 2000). If mutated, each of these genes does not cause a relevant phenotype, but the *sep1 sep2 sep3* triple mutant is similar to that of class B and C double mutants, such as *pi ag* and *ap3 ag*: the flower is composed only of concentric whorls of sepals. This demonstrates that class E genes genetically interact with class B and C genes to determine petal, stamen and carpel development.

Apart from AP2, all the ABCE class proteins are transcription factors belonging to the MADS box family.
The model described for *Arabidopsis* could be partially valid for *Antirrhinum*, although class A and E functions have not been corroborated yet.
A and C functions are mutually antagonistic; the A function excludes the C function from whorls 1 and 2 (so-called cadastral function) and the C function excludes the A function from whorls 3 and 4 (Bowman et al., 1989; Bowman et al., 1991)

1.1.3 The MADS box family

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

1.1.3.1 MADS box protein structure

In *Arabidopsis*, there are more than 100 MADS box genes (Alvarez-Buylla et al., 2000; de Bodt et al., 2003; Parenicova et al., 2003). The MADS box family can be divided into two groups based on their molecular domain structure: type I and type II.
The two groups differ in the amino acid consensus sequence of the MADS box domain and in the protein domain structure. Type I MADS box proteins in plants resemble the animal serum response factor (SRF) in their MADS domain, and their structure is more similar to that of animal and yeast MADS box proteins. Type I MADS box proteins have not yet been as extensively studied as the type II proteins.
The type II proteins include all homeotic MADS box factors and the vast majority of plant MADS box proteins of known function. Type II MADS box proteins have a characteristic domain structure. The MADS domain is located at the N-terminal end and mediates DNA binding, nuclear localization, and dimerization functions. Its amino

acid composition resembles the MADS domain of the *Drosophila* MADS box protein Myocyte-specific enhancer factor 2 (MEF2) (McGonigle et al., 1996; Riechmann and Meyerowitz, 1997; Immink et al., 2002). A second conserved domain, the K domain, mediates protein–protein interactions and dimerization (Fan et al., 1997; Yang et al., 2003a). In a subset of plant MADS box proteins, like AP1 and SEP3, the C terminal domain mediates a transcriptional activation function (Moon et al., 1999; Honma and Goto, 2001). The C terminal domain also has been reported to play a role in the formation of higher order MADS box complexes (Egea-Cortines et al., 1999; Honma and Goto, 2001).

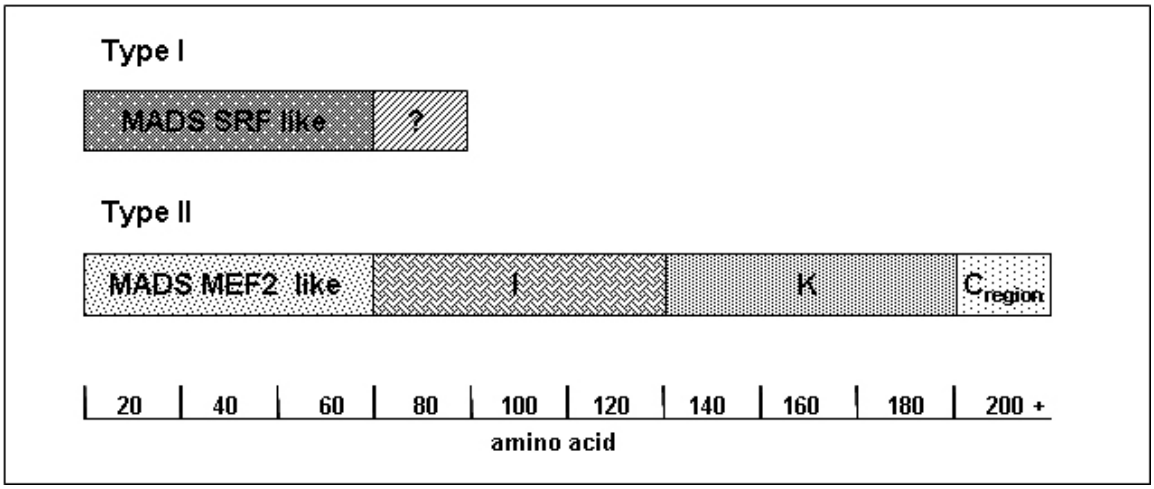


Figure 2. Structure of plant MADS box proteins.
MADS box proteins in plants were classified into two categories: the type I has a structure similar to MADS box proteins from human or yeast, and the MADS domain resembles that of SRF; the type II proteins have a modular structure, characteristic of plant MADS box proteins: the MADS domain, similar to that of MEF2, is followed by an Intervening (I) domain, a K domain and a C-terminal domain.

1.1.3.2 MADS box factors bind the DNA on CArG boxes

MADS box homeotic transcription factors guide the development of floral organs by interacting with the promoter of target genes, and regulating their transcription. The interaction of MADS box proteins with a cis-acting promoter element, named CArG box, is mediated by the MADS domain. To bind the DNA the MADS box proteins have to homo- and/or heterodimerize. Studies to identify the minimal DNA binding domain of the *Antirrhinum* MADS box proteins SQUAMOSA (SQUA) and PLENA (PLE) demonstrated that the MADS and I domains are sufficient to permit sequence-specific DNA binding by these proteins (West et al., 1998).

Similar results were obtained for the *Arabidopsis* MADS box proteins AP1, AP3, PI, and AG. In the case of AP3 and PI, that bind the DNA only as a heterodimer, the regions involved in the protein-DNA complex formation are the MADS domain, the entire I region and the first putative amphipathic helix of the K box. For AP1 and AG, that can bind the DNA as a homodimer, only the MADS box and part of the I region is needed (Riechmann et al., 1996a, b).

The sets of sequences recognized by AP1, AP3-PI and AG dimers *in vitro* are largely overlapping, suggesting that they achieve their functional specificity through selective interactions with additional transcription factors (Riechmann et al., 1996a, b).

The DNA interaction with MADS box proteins is often accompanied by DNA bending, which is thought to be a key determinant of their function as it is important in determining the local promoter architecture, by a yet unclear mechanism (Riechmann et al., 1996b; West and Sharrocks, 1999).

1.1.3.3 MADS box ternary interaction

MADS box homeotic transcription factors have been shown to interact with each other genetically. The interactions have been schematized in the ABCE model. Recently, evidences from *Arabidopsis* and *Antirrhinum* proposed that these interactions occur at a physical level as well.

Egea Cortines et al. (1999) showed that the capacity of DEF and GLO to bind the DNA *in vitro* (Tröbner et al., 1992) can be enhanced by a third MADS box factor, SQUAMOSA (SQUA) that forms a homodimer (Figure 3). This indicated, for the first time, that MADS proteins interact with the DNA as quartets.

According to the quartet model two dimeric forms of MADS box proteins would interact each with a CArG box, thereby causing DNA looping and triggering local changes in chromatin structure (Theissen and Saedler, 2001; Figure 3).

Honma and Goto (2001) corroborated this hypothesis with *in vivo* and *in vitro* experiments in *Arabidopsis*: AP3, PI, AP1 and SEP3 form ternary complexes in yeast. The co-expression of AP3 and PI together with either AP1 or SEP3, or both, is sufficient to drive the expression of a GUS reporter gene fused to the *AP3* promoter.

Furthermore, constitutive expression of AP3, PI, AP1 and SEP3 is sufficient to convert caulin leaves into petals, suggesting that this four proteins physically interact in the activation of the petal developmental program (Honma and Goto, 2001).

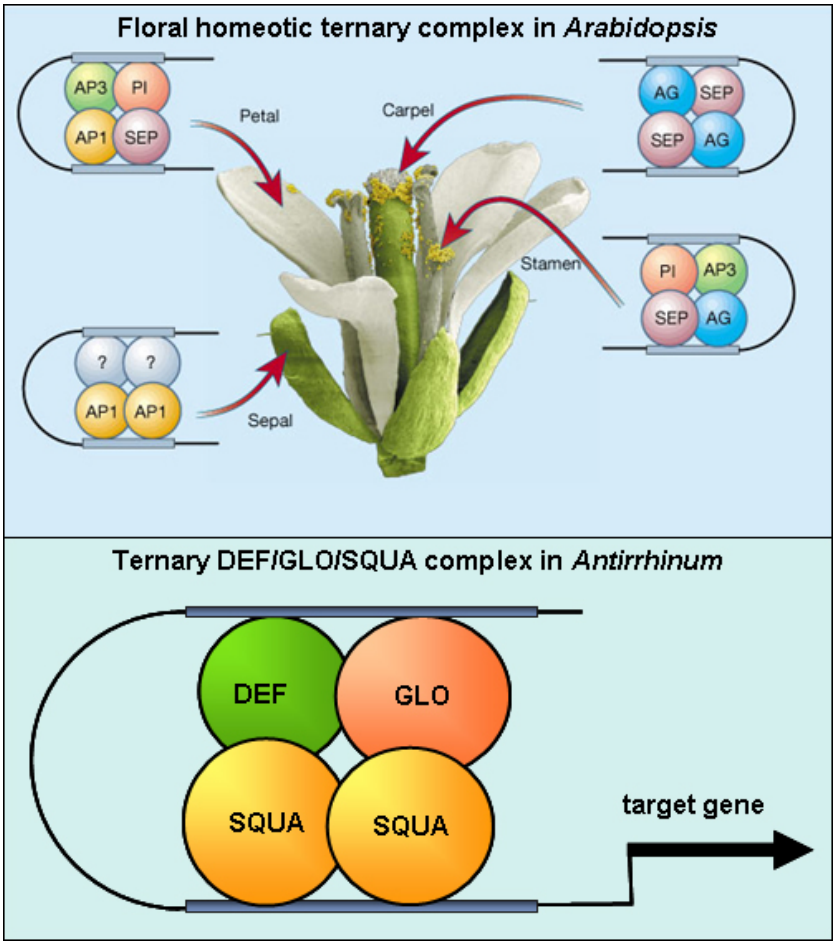


Figure 3. Formation of MADS box protein ternary complex in *Arabidopsis* and *Antirrhinum*.
A) Floral homeotic MADS box transcription factors, postulated to interact genetically in the determination of the development of floral organs, were shown to interact also physically (Honma et al., 2001) (picture from Theissen and Saedler, 2001).
B) *Antirrhinum* MADS box transcription factors were shown to bind the DNA as quartets (Egea-Cortines et al., 1999).
Two MADS box dimers would interact each with a CArG box (grey rectangle), causing DNA looping.

1.1.4 Class B activity in *Arabidopsis* and *Antirrhinum*

The class B homeotic genes in *Antirrhinum* and *Arabidopsis* are *DEFICIENS* (*DEF*) and *GLOBOSA* (*GLO*), and *APETALA3* (*AP3*) and *PISTILLATA* (*PI*), respectively. Analysis of their amino acid sequences suggested that PI and GLO, and DEF and AP3, are homologous.

A mutation in any of these four genes (*def*, *glo*, *ap3*, *pi*) causes a homeotic conversion of petals into sepals. In *Arabidopsis,* the *ap3* and *pi* mutant flowers develop carpels in the third and fourth whorls. In *Antirrhinum*, in the center of *def* and *glo* mutant flowers one single whorl develops, containing five locules fused together in a tubular structure, instead of two whorls, occupied by stamens and a bilocular gynoecium (Schwarz-Sommer et al*.,* 1992; Tröbner et al*.,* 1992; Zachgo et al*.,* 1995).

The transcripts of *DEF* and *GLO* are first detected in young flower buds, when sepal primordia initiate, in the area where petals and stamens will arise. *DEF* is also expressed in the center of the flower. The expression of *AP3* at stage 3 (according to Smyth et al*.*, 1990) is similar to that of *GLO*, i.e. restricted to second and third whorls, whereas the expression of *PI* resembles that of *DEF*, e.g. in the three inner whorls (Goto and Meyerowitz, 1994; Jack et al*.* 1994; Schwarz-Sommer et al*.,* 1992; Tröbner et al*.,* 1992).

At later stages of development, when organ primordia are formed, *DEF* and *GLO* transcripts are maintained at high levels in petals and stamens, and at much lower levels in the gynoecium. *DEF* and *GLO* are strongly expressed until flowers are fully developed. In spite of their strong expression throughout stamens, *DEF* and *GLO* transcripts are not detectable in developing pollen.

In *Arabidopsis*, *PI* transcript disappears from the center of the bud as soon as organ primordia are initiated and is detected only in petals and stamens, until the flower is mature. From stage 3 on, *AP3* is expressed in petals, stamens and ovules (Goto and Meyerowitz, 1994; Jack et al*.*, 1994).

The expression of class B genes in class B mutants, both in *Arabidopsis* and in *Antirrhinum*, is initiated as in the wild type, but is reduced in later stages, when organ primordia are distinguishable on the floral meristem (Goto and Meyerowitz, 1994; Schwarz-Sommer et al*.,* 1992; Tröbner et al*.,* 1992).

These data suggested that class B genes are able to maintain their own expression by an autoregulatory loop. DEF and GLO bind, as a heterodimer *in vitro*, CArG boxes present in their own promoter, indicating that they probably directly promote their transcription (Tröbner et al*.,* 1992).

AP3 and PI are also able to bind, as a heterodimer *in vitro*, two of the three CArG boxes in the *AP3* promoter. They maintain the transcription of *AP3* by interacting directly with its promoter, in third whorl organs. In second whorl organs, *AP3* is

maintained at high levels in *pi* mutants, but not in *ap3* mutants (Jack et al., 1994). Hence, the presence of AP3, but not of PI, is required for the maintenance of *AP3* transcription.

For the maintenance of *PI* transcription, both PI and AP3 are required in the second and third whorl. However, *PI* autoregulation probably is indirect, because it requires *de novo* protein synthesis, and the fragment of the *PI* promoter shown to be essential for the *PI* maintenance does not contain CArG boxes and is unable to bind the AP3/PI heterodimer *in vitro* (Chen et al., 2000; Honma and Goto, 2000).

DEF was shown to be able to replace the AP3 function in *Arabidopsis*: 7kbp *DEF* genomic locus could complement the *ap3* mutant phenotype in an heterologous system called *ap3*TDEF. In *ap3*TDEF flowers, the *DEF* expression domain, with respect to that of *AP3*, is extended to the inner whorl. The coexpression of DEF and PI in the center of the flower triggers the maintenance of *PI* transcription, and leads to the ectopic development of stamens (Samach et al., 1997).

1.1.5 Class B target gene investigation

Floral homeotic transcription factors determine the development of floral organs by regulating the expression of different sets of target genes. Theissen and Saedler (2001) proposed that MADS box homeotic proteins, promoting the formation of the same organ, interact as quartets to regulate the expression of overlapping sets of target genes (Figure 3).

Therefore, in order to understand the development of floral organs, the isolation of target genes is crucial. This is especially relevant for class B transcription factors that activate very different developmental programs, like the formation of petals and stamens.

In the past decade, several studies were carried out to identify class B transcription factor target genes. The first putative target gene was identified by Sablowski and Meyerowitz (1998), using an inducible system coupled with differential display (Liang et al., 1992): the AP3-Glucocorticoid Receptor (GR) fusion protein, constitutively expressed in *Arabidopsis,* is able to enter the nucleus and activate target genes only upon dexamethasone treatment. The dexamethasone treatment was coupled with a cycloheximide treatment, to block the protein production in the cell and to ensure that

the AP3-GR activated genes are direct targets of AP3 that and no other proteins mediated their activation. Comparing cDNA obtained from treated and mock-treated flowers by differential display, the authors could isolate a gene called *NAC-LIKE, ACTIVATED BY AP3/PI* (*NAP*), that turned out to be expressed within the AP3/PI activity domain and not in vegetative tissues. The expression of *NAP* was reduced in *ap3-3* mutants, supporting the idea that *NAP* might be a target gene of AP3.

The same inducible system was used more recently by Zik et al. (2003) to identify genes regulated by AP3, by comparing dexamethasone treated and mock-treated inflorescences by DNA microarray. The authors estimate that the number of genes controlled by AP3/PI is around 200. This set of genes includes cell wall–associated genes, genes involved in stress response, as well as genes related to cell signaling and transcriptional regulation.

The two studies cited here do not bring evidence for a direct interaction of AP3 with their promoters, and do not prove that these genes participate in the petal and stamen developmental program.

The GR/dexamethasone inducible system was exploited by Gómez-Mena et al. (2005) to investigate the target genes of AGAMOUS (AG). Microarray chips were hybridized with cDNA prepared from *Arabidopsis* transgenic plants, expressing the AG-GR fusion protein, treated with dexamethasone or a mock solution.

The set of AG target genes identified was poorly overlapping with the set of genes that was proposed by Zik et al. (2003) as targets of AP3. This would suggest that most of the genes regulated by B and C class transcription factors could not be identified by these two approaches, since B and C class proteins are thought to physically interact, regulating target genes in the stamen development (Theissen and Saedler, 2001).

Wellmer et al. (2004) compared by microarray the expression profile of homeotic mutants that do not produce petals (*ap3*, *ap1* and *ap2*) or do not produce petals and stamens (*ap3*) or do not produce stamens and carpels (*ag*). By combining these data, they were able to isolate sets of organ specific genes. For instance, they found that only 18 genes are petal specific and >1100 are stamen specific.

In order to produce a transcriptional profile of *Antirrhinum* petals, and detect possible targets of DEF and GLO, Bey et al. (2004) hybridized macroarray filters carrying over 11.000 unigenes with cDNA from petals at four developmental stages. Furthermore,

they exploited the temperature inducible system *def-101* (Zachgo et al*.,* 1995), where the DEF protein is active at 15°C but inactive at 26°C, to isolate targets of DEF: the cDNA of petals at the stage of most intensive growth (stage 3) from plants grown at 15°C was compared, by macroarray, to that of petals from plants grown at 15°C, but shifted to 26°C for 24 and 72 hours before collecting material.
The differentially expressed genes (about 200) cover a broad variety of functions, similarly to what reported by Zik et al*.* (2003). However, only a few genes had a similar function, like a GDSL-lipase, that was indicated as a possible target in both studies.

1.2 Strategies to isolate direct target genes

Differential display, microarray and a few other techniques were described as a possible approach for the isolation of transcription factor target genes *in vivo* (Bey et al., 2002). Among these the DamID technique was applied for *Drosophila* by Steensel et al*.* (2000): the transcription factor that directly interacts with the DNA is expressed as a fusion to the *Escherichia coli* DNA adenine methyltransferase (Dam). This fusion protein will methylate the adenine on the genomic DNA surrounding the binding site of the protein under investigation. Genomic DNA is then extracted from *Drosophila* cell lines expressing the fusion protein and digested by a methyl sensitive restriction enzyme, e.g. Dpn I that cuts the DNA on the GATC site only when the A is methylated. The small fragment fraction (~200-500bp) that should be enriched in target sequences, is then separated from large and uncut genomic fragments, and hybridized to microarrays. Unspecific methylation background is determinated by control hybridization, conducted with material extracted from cells expressing the Dam alone.
In spite of being a promising *in vivo* approach for the study of target genes, the DamID has not been extensively used, most probably for the high toxicity of the Dam enzyme.
The Crosslink Chromatin Immunoprecipitation (X-ChIP) technique is a much more successful approach (Orlando et al., 1997; Spencer et al*.,* 2003). In this technique,

proteins are *in vivo* covalently linked to the DNA by paraformaldehyde (PFA), then nuclei are extracted and the chromatin is sheared by sonication. The DNA fragments bound by the protein of interest are isolated with a specific antibody.

The X-ChIP technique has been first used in works performed in yeast and animals (Orlando et al., 1997; Spencer et al*.,* 2003). Recently, the X-ChIP technique has been applied to the study of plant transcription factors (Wang et al*.,* 2002; Dilusha et al*.,* 2004; Johnson et al*.,* 2001; Zhang et al., 2001; Gómez-Mena et al*.*, 2005).

1.3 Floral organ initiation, the role of ROXY1

The class ABCE homeotic genes control the identity of organ primordia, but do not determine their number and position. These processes are controlled by another group of genes, expressed at very early stages of development on the floral meristem.

In *Arabidopsis*, initiation of petal primordia is altered in mutants like *perianthia* (*pan*), where 5 petals and 5 sepals develop (Running et al*.,* 1996). In other mutants, like *petal loss* (*ptl*) or *rabbit ears* (*rbe*), the number of petals is reduced (Brewer et al*.,* 2004; Takeda et al*.,* 2004). Both *PTL* and *RBE* encode transcription factors that are expressed on the floral meristem between sepal primordia and in petal primordia precursor cells, respectively.

roxy1 is a newly characterized mutant that shows alteration in petal initiation and development (Xing et al*.,* 2005). *roxy1* mutants form only 2,5 petals instead of 4, that often show an aberrant development at late developmental stages.

ROXY1 is also supposed to be a negative regulator of *AGAMOUS*, since the expression of *AG* is enhanced in *roxy1* double mutants with AG repressors, like *ap1* or *ap2*.

The *ROXY1* gene encodes for a glutaredoxin. Glutaredoxins (GRXs) are small proteins found in many prokaryotic and eukaryotic organisms and have been shown to participate in redox reactions.

1.3.1 ROXY1 encodes a glutaredoxin

GRXs are small redox proteins of the thioredoxin (TRX) superfamily. GRXs are involved in reactions that reduce disulfides via a monothiol or dithiol mechanism (Figure 4).

The reaction involves conserved sites that contain two cysteines (CXXC) in the dithiol mechanism, or one cystein (CXXS) in the monothiol mechanism. The dithiol mechanism can reduce both protein disulfides and glutathione (GSH) mixed disulfides, whereas the monothiol mechanism can only reduce glutathione (GSH) mixed disulfides (Lemaire, 2004).

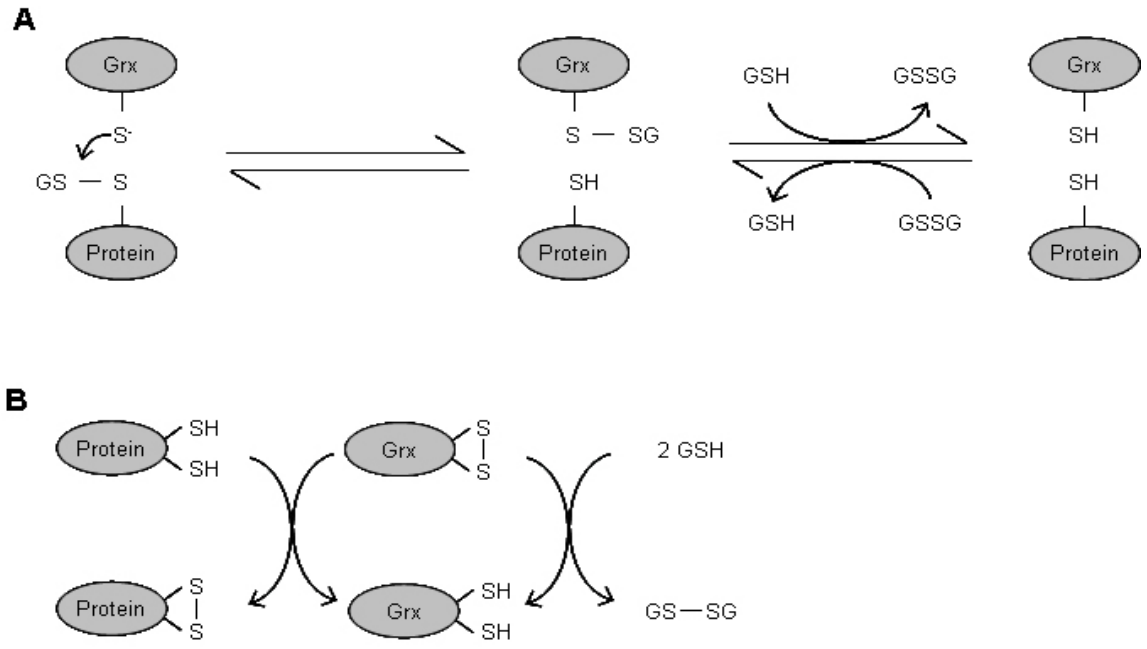


Figure 4. Posttranslational modification mediated by ROXY1 via the dithiol and monothiol mechanism.
A) Glutathione (GSH) mixed disulfides reduction through the monothiol mechanism. Arrows indicate a glutathionylation reaction (progressing from right to left) and a deglutathionylation (progressing from left to right).
B) Oxidation of disulphide bridges through the dithiol mechanism. Two examples are reported: the oxidation of intramolecular disulphide bridges of a target protein and the oxidation of two glutathiones to a glutathione dimer.

Both these reactions posttranslationally modify proteins. The addition (glutathionylation) or removal (deglutathionylation) of GSH from cystein residues of target proteins is a reversible reaction that can mediate their inactivation or activation.

Generally, glutathionylation is associated with an inactivation of the protein, whereas the deglutathionylation reactivates the protein (Nulton-Persson et al., 2003; Lillig et al., 2003; Ito et al., 2003; Fratelli et al., 2002).

GRXs or TRXs, and glutathione can promote the nuclear translocation and the DNA binding activity of human transcription factors, such as nuclear factor (NF) ĸB and activator protein (AP) -1 (Sen and Packer, 1996).

ROXY1 is the only plant glutaredoxin family member that has been characterized to date. Other plant GRXs have putative roles in calcium transport or are supposed to regulate, by glutathionylation/deglutathionylation, the activity of enzymes like triosephosphate isomerase or aldolase (Cheng et al., 2003; Ito et al., 2003).

ROXY1 could be involved in the activation/inactivation of target proteins by the removal/addition of GSH through its CXXS or CXXC active sites. ROXY1 could as well participate in the posttranslational modifications of target protein secondary structure by mediating the formation of dithiol bridges, which would involve the CXXC active site.

Therefore, in order to clarify how ROXY1 influences the petal primordia initiation, isolating its target proteins and understanding how these proteins participate in the floral development is essential.

1.4 Posttranslational modifications and gene transcription regulation

ROXY1 could be involved in a posttranslational modification of target proteins, by mediating the formation of intermolecular dithiol bridges or by glutathionylation. Other types of posttranslational modifications are known to reversibly regulate the activity of many different proteins, enzymes, transcription factors, histones, and are known to influence organ development in many organisms, including plants (Huber and Hardin, 2004).

Protein kinases are enzymes responsible for the addition of a phosphate to serine, threonine or tyrosine residues (phosphorylation). The reaction can be reversed by a phosphatase (dephosphorylation). Protein phosphorylation is the major control

mechanism of diverse cellular processes including cell division, protein synthesis and transcriptional regulation. For instance the phosphorylation of a serine residue can influence the DNA binding capacity of the MADS box protein MEF2 (Molkentin et al*.,* 1996), or regulate the synthesis of mRNA by RNA polymerase II (Kobor et al*.,* 2002). The *TOUSLED (TSL)*-like nuclear protein kinase family is involved in both leaf and flower development, and *tsl* loss of function mutations cause abnormal growth and initiation of floral organ primordia. TSL-mediated histone phosphorylation may be involved in gene repression and/or chromatin regulation in *Arabidopsis* (Ehsan et al*.,* 2004).

The posttranslational modification of histones plays a central role in chromatin density modification, and repression or activation of the genes that interact with the modified histones. Generally, histone acetylation is coupled with decreased chromatin density and gene activation, whereas histone deacetylation or methylation is accompanied with gene silencing (Reyes et al*.,* 2002; Tian and Chen 2001).

For instance, a negative regulator of *AGAMOUS* (*AG*), LEUNIG (LUG) is highly similar to a *Drosophila melanogaster* protein, Groucho, that has a transcriptional repressing activity through the interaction with the histone deacetylase Rpd3 (Conner et al*.,* 2000; Chen et al*.,* 1999).

It has been postulated that histone deacetylation is followed by histone methylation, that prevents their re-acetylation. Polycomb Group (PcG) proteins are involved in complexes that mediate histone methylation. The PcG protein MEDEA (MEA) is responsible for the negative regulation of the MADS box gene *PHERES1* (Köhler et al*.,* 2003), and the PcG protein CURLY LEAF (CLF) is a negative regulator of *AG* (Goodrich et al*.,* 1997), suggesting that PcG proteins act as negative regulators of MADS box genes in *Arabidopsis*.

1.5 Goal of the work

In the work presented here an effort is conducted to understand the molecular mechanisms of petal initiation and development.

The interaction of class B transcription factors AP3 and DEF with putative target sequences is investigated by X-ChIP experiments. A new approach to the data analysis is elaborated, in order to discern false positives from real interactions. Furthermore, homeotic MADS box protein interactions are investigated by in yeast assays: AP3 and PI were employed as baits in a three hybrid screening.

Finally, putative interactors of ROXY1 are isolated by a yeast two-hybrid screening and their expression is characterized by *in situ* hybridization.

2. MATERIALS AND METHODS

2.1 Chemicals, enzymes and oligonucleotides

Chemicals used for these experiments were purchased from the following companies: Life Technology Pharmacia (Freiburg), Sigma (Deisenhofen), Merck (Darmstadt), Biomol (Hamburg), Fluka (Neu-Ulm) and Promega (Madison). Nylon membranes were obtained from Amersham (Braunschweig).

Enzymes were purchased from Roche (Mannheim), Biolabs (England), Life Technology (Freiburg), MBI Fermentas (St. Leon-Rot), Pharmacia (Freiburg) and Sigma (Deisenhofen).

10 x buffers were supplied together with the corresponding enzymes.

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

2.2 Plant materials

Antirrhinum wild-type lines 165E and mutant *def-gli* plants were grown at 18-25°C (16 hr light/8 hr dark) in greenhouse.

Arabidopsis wild type Columbia and mutant *ap3-1* were also grown in the greenhouse in longday (16 hr light/8 hr dark) and short-day (10 hr light/14 hr dark) conditions.

2.3 Bacterial strains

Escherichia coli

DH10B F min, *mcr*AD (*mrr-hsd*RMS-*mcr*BC) F80*lac*ZDM15, D*lac*X74, *deo*R, *rec*A1, *end*A1, *ara*D139, D (*ara, leu*)7607, *gal*U, *gal*K, I min *rps*1, *nup*G.

Agrobacterium tumefaciens
GV3101 (Van Larabeke et al., 1974)

2.4 Yeast strains

PJ69-2A *MATa, trp1-901, leu2-3, 112, ura3-52, his3-200, gal4D, gal80D, LYS2::GAL1 UAS-GAL1 TATA-HIS3, GAL2 UAS-GAL2TATA-ADE2, MEL1* (Clontech)
Y187, *MATa, ura3- 52, his3- 200, ade 2- 101, lacZ, & trp 1- 901, leu 2- 3, 112, gal4D, met–, gal80D, URA3 :: GAL1UAS -GAL1TATA -lacZ, MEL1* (Clontech)
AH109 *MATa, trp1-901, leu2-3, 112, ura3-52, his3-200, gal4D, gal80D, LYS2 : : GAL1UAS-GAL1TATA-HIS3, MEL1 GAL2 UAS-GAL2TATA-ADE2, URA3::MEL1 UAS-MEL1TATA-lacZ*

2.5 Cloning vectors

pBluescript KS (+) (Stratagene)
pGADT7 and pGADT7-Rec (Clontech)
pGBKT7 (Clontech)
pGEM-T (Promega)
pBARA (derivative of pGPTV-BAR, Becker *et al.*, 1992)

2.6 The X-ChIP technique

2.6.1 Plant material fixation

Three to Five grams of freshly harvested tissue is incubated with 45 ml of fixation buffer (0,1 M Na-Phosphate buffer pH 7,4; 1% Paraformaldehyde) for 20 min under vacuum and for 1 hour and 40 min on a rotating wheel at 4°C, for *Antirrhinum,* and 45 min under vacuum for *Arabidopsis*. For *Arabidopsis* 100 µl of tween-20 are added to the fixation buffer. The fixation reaction is stopped by adding glycine to the final concentration of 0,125 M for 5 min at RT. Samples are washed three times with

washing buffer (0,1 M Na-Phosphate buffer pH 7,4; glycine 0,125 M). Buffer is discarded and samples are frozen in liquid nitrogen and stored at -80°C.

2.6.2 Immunoprecipitation

Plant tissues are ground in liquid nitrogen. Powder is collected and homogenized in 30 ml buffer A (10 mM HEPES/KOH, pH 7,9, 10 mM KCl, 1,5 mM MgCl, 0,5 mM DTT, 0,1% Nondinet P-40 substitute, 10% glycerol) with 200 µl proteinase inhibitor (Sigma P-9599), and filtered through 300 – 75 – 20 and 10 µm membranes. Nuclei are collected by centrifugation at 1700 rpm for 10 min, and then washed 5-10 times with 5-10 ml buffer A. Nuclei are resuspended in 1,5 ml of buffer A and transferred to a 1,5 ml Eppendorf tube. Nuclei are pelleted by at 500 g at 4°C for 5 min and resuspended in sonication buffer (10 mM HEPES/KOH, pH 7,9, 100 mM NaCl, 1 mM EDTA, pH 8,0, 1 mM EGTA, pH 8,0, 0,1%Triton X-100, 0,1% sodium deoxycholate, 125 mM glycine). Chromatin is fragmented using a Hielscher UP50H sonicator (dr. Hielscher GmbH, Germany) with an amplitude setting of 60% and a cycle control of 0,65 for 20 times 10 seconds. Nuclear debris are pelleted by centrifugation at 13000 rpm for 5 min. A 10-20 µl aliquot of the supernatant is taken: this is the “Input DNA”, used for SQ-PCR primer normalization (see results).

The supernatant is added to an equal volume of 2xRIPA buffer (50 mM HEPES/KOH, pH 7,9, 180 mM NaCl, 1 mM EDTA, pH 8,0, 1% Nondinet P-40 substitute, 0,2% sodium deoxycholate, 125 mM glycine, 0,05% SDS and proteinase inhibitor cocktail (Sigma P-9599, 1 ml per 150 ml buffer)). Samples are incubated with 4 µl of pre-immune serum at 4°C for 1h on a rotating wheel. 40 µl protein A-sepharose beads (Pierce, USA) were washed 4 times in 1xRIPA buffer (50mM HEPES/KOH, pH 7,9, 140mM NaCl, 1mM EDTA, pH 8,0, 0,5% Nondinet P-40 substitute, 0,1% sodium deoxycholate, 125 mM glycine, 0,025% SDS and proteinase inhibitor cocktail (Sigma P-9599, 1 ml per 150 ml buffer), added to the reaction and incubated on a roller at 4°C for 1 hour. Protein A sepharose beads were accumulated by centrifugation at 13000 rpm, 4°C, for 2 minutes, the supernatant was mixed with 4 µl of anti-DEF serum and incubated at 4°C on a roller overnight.

For GFPC and 6MN plant material the pre-absorption step with pre-immune serum was skipped and samples were directly incubated either with 50 µl anti-GFP serum (eBioscience, 14-6774-63) or anti-c-MYC monoclonal antibody (Sigma, M-5546).
40 µl of prewashed protein A sepharose beads were added to the immunoprecipitation reaction and incubated at 4°C on a roller for 2 hours. Immunoprecipitated protein-DNA complexes were accumulated by centrifugation at 13000 rpm, 4°C for 2 min. Ice cold RIPA buffer were added to the beads and incubated on a roller at room temperature for 10 min. Beads are pelleted by centrifugation at 13000 rpm, room temperature, for 1 min the supernatant is discarded and the washing step is repeated three times. Beads are pelleted by centrifugation and the immunoprecipitated protein-DNA complexes are eluted from the protein A beads by the addition of 100 µl glycine elution buffer (0,1 M glycine, 0,5M NaCl, 0,05% Tween-20, pH 2,8), vortexing for 15 seconds and centrifugation at 13000 rpm, room temperature, for 1 min. The supernatant is mixed with 50 µl 1 M tris pH 8,0 and the elution is repeated twice. The eluted fractions are pulled together, centrifuged at 13000 rpm, room temperature, for 2 min and 420 µl of the supernatant are transferred into a fresh Eppendorf tube. Residual RNA is degraded by the addition of 40 µg RNase A and incubation at 37°C for 30 min. SDS is added to a concentration of 0,25% and proteins are digested by adding 750 ng/ml Proteinase K and incubation 37°C overnight. Formaldehyde-induced cross-links are reversed by incubation at 65°C for 6 hours. The reaction is adjusted to binding conditions by the addition of 215 µl isopropanol, 0,07 g guanidine chloride and 7 µl 2 M NaOAc, pH 4,0, loaded onto a Qiagen MiniElute column (Qiagen, Germany), centrifuged at 13000 rpm, room temperature for 1 min, washed twice with buffer PE and eluted with 12,5 µl 5mM tris pH 8,0. The elution is performed twice. The DNA yield is 1-10 ng.

2.6.3 Semi Quantitative-Polymerase Chain Reaction (SQ-PCR)

About 1/10 of the eluted Immunoprecipitated DNA (IPed DNA) is used per each Semi Quantitative-Polymerase Chain Reaction (SQ-PCR). The SQ-PCR is performed in a 25 µl volume (0,5 µl each 10 µM primer, 0,5 µl 10 mM dNTPs, 2,5 µl 10x ammonium buffer, H_2O to 25 µl) for 33 cycles (94°C denaturating temperature 50 sec; 58°C annealing temperature 50 sec, 72°C extension temperature 30 sec), using the

Ampliqon taq enzyme with ammonium buffer (Ampliqon, Denmark). Five µl 6x ficoll loading buffer is added to the SQ-PCR product and 20 µl of the resulting mix is loaded on a Et/Br 1,2% Agarose gel. In order to quantify bands intensities, gels are scanned with a Phopshoimager Scanner (Molecular Dynamics) and gel pictures are analyzed by Image Quant (Molecular Dynamics).
Average, standard deviation and t student are calculated with MS Excel (MicroSoft).

X-ChIP primers

GLOBOSA
Region 1 AL1105 AL1104
Region 2 AL1121 AL1122
Region 3 X22 X23
Region 4 AL1123 AL1124
Region 5 AL1125 AL1126

DEFICIENS
Region 1 X16 AL428
Region 2 AL1028 AL1030
Region 3 AL482 AL483

DEFH125
Region 1 750 751
Region 2 748 749
Region 3 AL1190 AL1191
Region 4 152 747
Region 5 AL1194 AL1195

EXTENSIN
Region 1 AL1081 AL1083
Region 2 AL1084 AL1085
Region 3 591 740
Region 4 733 AL811

Region 5 AL1109 AL1110
Region 6 AL1077 AL1080

Double immunoprecipitation library clones
Clone 1 AL1144 AL1145
Clone 11 AL1148 AL1149
Clone 14 AL1150 AL1151
Clone 18 AL1152 AL1153
Clone 21 AL1154 AL1155
Clone 23 AL1156 AL1157
Clone 28 AL1158 AL1159

2.6.4 anti-DEF serum purification

Unspecific antibodies contained in the anti-DEF serum were depleted by running the serum through HiTrap NHS-activated HP colums (Amersham Biosciences). Manufacturer's instructions were followed.

2.7 Enzymatic modifications of DNA

2.7.1 Digestion with restriction enzymes

Digestion with restriction enzymes was performed according to the supplier's recommendations and in the provided buffers. The digestion was carried out for 1-2 hours with 10 units of enzyme at an appropriate temperature (mostly at 37°C), and stopped by heat inactivation at 75°C for 10 min.

2.7.2 Ligation of DNA fragments

Ligation of DNA fragments to a linearized plasmid vector involves the formation of new bonds between a phosphate residue located at the 5´ termini of double-stranded DNA and a 3.- hydroxyl moieties. To avoid recircularisation of the vector, the 5.

terminal ends were dephosphorylated using Calf Intestinal Phosphatase (CIP, NEB). The ligation reaction contained 1 mM of ATP and 5% of PEG 8000. The insert was also sometimes incubated at 65°C for 5 min before adding T4 DNA ligase. Ligation was performed for at least 4 hrs at 12-16°C.

2.8 Bacterial transformation

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

Basically, the bacteria were grown to mid-log phase, chilled, centrifuged, and then washed extensively in sterile conditions to reduce the ionic strength of the cell suspension. A single colony was inoculated into 10 ml of LB media and cultured overnight. This overnight culture was used to inoculate a 500 ml culture in the next morning, and grown at 18°C till the OD at 600 nm reached 0.4. Freshly grown bacterial cells were centrifuged down at 4,000 g for 15 min at 0°C, resuspended with 500 ml of ice-cold distilled water. Resuspended cells were washed again with 250 ml of ice-cold distilled water after centrifugation. Finally, the cells were washed with 20 ml of distilled water, centrifuged down, resuspended in 1ml of distilled water containing 7% DMSO. The cells were aliquoted in 100 µl in sterile tubes, quickly frozen in liquid nitrogen and stored at -70°C.

2.8.2 Electroporation of bacterial cells

Frozen electro-competent cells were thawed on ice and mixed with 2 µl of ligation mix. The mixture was transferred into a prechilled cuvette. The electroporation was done at 1800 V. A single electroporation pulse was given, and 1 ml of SOC media immediately added. After incubation at 37°C (or at 28°C for the *Agrobacterium*) for 1 hour, the cells were plated onto selective media. SOC media: 2% Bacto tryptone, 0.5% Bacto yeast extract, 10 mM NaCl, 250 mM KCl, 10 mM MgCl2, 10 mM MgSO4, 20 mM glucose (filter sterilized, added whenever it is needed).

2.9 Yeast two-hybrid screening

2.9.1 Plasmid constructions for two-hybrid screening

p3HS1: the coding sequence of *AP3* was obtained by PCR with primers AL257 and AL258 from the vector p258A (gift of Dr. Simona Masiero). PCR product was cut on the EcoRI and SalI restriction site artificially introduced in the primer by the respective restriction enzyme, and ligated in the pBRIDGE vector (Clontech) cut open with the same restriction enzymes.

p3HS2: the coding sequence of *PI* was obtained by PCR with primers AL303 and AL304 from the vector p122 (gift of Dr. Susanna Schwartz-Sommer). The PCR product was cut on the NotI and BglII restriction site artificially introduced in the primer by the respective restriction enzyme, and ligated in the pBRIDGE vector (Clontech) cut with the same restriction enzymes.

p3HS3: the fragment cut from p3HS1 by EcoRI and SalI was cloned in p3HS2 cut by EcoRI and SalI.

p2B and p2H: the three point ligation was performed ligating a first insert, the *URA3* coding sequence amplified from the vector pLacZi (Clontech) by AL797 and AL827, with the second insert, the *met* promoter of pBRIDGE (clontech) cut by NotI and NcoI and the vector: the pBRIDGE, for p2B, or p3HS2, for p2H.

pS2B; pL2B and pL2H: the PCR fragment amplified from p3HS3 with AL868 and AL867 was cut with HindIII; the shorter fragment was ligated into p2B cut with HindIII obtaining pS2B; the longer fragment was ligated into p2B cut with HindIII obtaining pL2B and into p2H cut with HindIII obtaining pL2H.

pAP3E and pAP3PI: the AP3 coding sequence was cut from p3HS1 by EcoRI and SalI and ligated to the *URA3*-containing fragment cut from pL2B, for pAP3E, or from pL2H, for pAP3PI, by SalI and BglII, and ligated into the vector pS2B cut with EcoRI and BglII.

pAPINLS: a four points ligation was performed ligating the *AP3* coding sequence amplified from p3HS1 by AL257 and AL893 and cut by EcoRI and SalI, with the *URA3*-containing fragment cut by SalI and NotI from pL2B, with the *PI* coding

sequence amplified from p3HS2 by AL303 and AL895 and cut by NotI and BglII, with the vector pS2B cut by EcoRI and BglII.

pGAP3: *AP3* coding sequence was cut from p3HS1 by EcoRI and SalI and cloned in pGADT7 cut by EcoRI and XhoI.

pBRIPI: *PI* coding sequence was amplified from p122 by AL255 and AL256 and cut by EcoRI and SalI and cloned in pTFT1 (Egea-Cortines et al, 1999) cut by EcoRI and SalI.

pGPI: *PI* coding sequence was cut by EcoRI and BglII from pBRIPI. Cloned in pGADT7 cut by EcoRI and BamHI.

pDEF1: *DEF* coding sequence amplified by AL939 and AL941 and digested with EcoRI and SalI. cloned in pAP3PI cut by EcoRI and SalI

pDEGL: *GLO* coding sequence amplified by AL943 and AL945 and cut by NotI and BglII. Ligated in pDEF1 cut by NotI and BglII.

pGADH: fragment cut from pAP3E by NsiI and EcoRI and cloned in vector pGADT7 cut by NsiI and EcoRI.

pCAD: fragment amplified by AL934 and AL935 from pGADT7, cut by XhoI and SalI and ligated in pGADH cut by XhoI.

pGSEP3 (gift of Ulrike Hartmann). *SEP3* coding sequence cloned in pGADT7 by NcoI and XhoI.

pSQAD: fragment amplified from plasmid pGSEP3 by AL947 and AL1003 cut by EcoRI and SalI and cloned in pCAD cut by EcoRI and XhoI.

pGLAD fragment amplified from pDEGL by AL1004 and AL1005 cut by EcoRI and SalI and cloned in pCAD cut by EcoRI and XhoI.

pDEFAD gift of Dr. Simona Masiero.

p2HAP3 200bp of *AP3* promoter amplified by AL802 and AL803, cut by SpeI and EcoRI, ligated in ycphis3bs cut by SpeI and EcoRI.

p8HAP3 800bp of *AP3* promoter amplified by AL804 and AL805, cut by SpeI and EcoRI, ligated in ycphis3bs cut by Spe I and EcoRI

pGLOp *GLO* promoter amplified by AL949 and AL950 cut by SpeI and EcoRI and cloned in ycphis3bs cut by SpeI and EcoRI

pDHp DEFH125 promoter amplified AL961 and AL962 cut by SpeI and EcoRI and cloned in ycphis3bs cut by SpeI and EcoRI

pR1 and pR2 the full length coding sequence was PCR amplified by AL1377 and AL1378 (pR1) AL1379 and AL1380 (pR2) and cloned in pGBKT7 by EcoRI and BamHI restriction sites

TCP3, TCP4, TCP14, TCP21, RBE, TGA3 SnRK the full length coding sequence was PCR amplified and cloned in pGADT7. Primers and respective restriction sites artificially introduced and used for the cloning are listed below.

TCP3 AL1444 AL1445 SalI/SalI

TCP4 AL1446 AL1447 SalI/SalI

TCP14 AL1487 AL1480 EcoRI/XhoI

TCP21 AL1450 AL1451 EcoRI/SalI

TGA3 AL1452 AL1453 EcoRI/SalI

RBE AL1463 AL1464 EcoRI/SalI

SnRK AL1477 AL1459 EcoRI/SalI

2.9.2 Small scale transformation in yeast

A single colony was scraped from a plate into 10 ml of appropriate selective medium and grown overnight at 30°C. The culture was diluted in the same medium according to the number of transformations (using 5 ml per transformation) and regrown for 2 hours. The final culture was pelleted and washed with water, 1 ml of 1 x LiAc/TE, and resuspended in 1 x LiAc/TE of 100 µl per transformation. The suspension was added to a mixture of 1 µg plasmid DNA, 10 µg salmon sperm carrier DNA, 300 µl PEG/LiAc/TE, and 5 µl DMSO. The mixture was first incubated at 30°C for 30 min, then at 42°C for 15 min and a 100 µl aliquot was plated on selective plates.

1x LiAc/TE 10x TE

10 x LiAc 100 µl 1 M Tris-HCl pH7.5 100 ml

10 x TE 100 µl 500 mM EDTA pH7.5 20 ml

Sterile water 800 µl add H_2O to 1 liter

1x PEG/LiAc/TE 10x Lithium acetate

10 x LiAc 500 µl Lithium acetate 40.8 g

10 x TE 500 µl add H_2O to 400 ml

50% (W/v) PEG 4000 4 ml Adjust to pH 7.5

2.9.3 Plasmid DNA isolation from yeast

Lysis solution: 10X Dropout

Triton X-100 2% L-Isoleucine 300 mg

SDS 1% L-Valine 1500 mg

NaCl 100 mM L-Adenine hemisulphate salt 200 mg

Tris pH 8.0 10 mM L-Arginine HCl 200 mg

EDTA 1 mM L-Histidine HCl monohydrat 200 mg

L-Leucine 1000 mg

YPD medium: L-Lysine 300 mg

Peptone/Tryptone 20 g L-Methionine 200 mg

Yeast Extract 10 g L-Phenylalanine 500 mg

Adjust to pH 5.8 L-Threonine 2000 mg

Add H20 to 950 ml and autoclave L-Tryptophan 200 mg

Before use add 50 ml of 40% dextrose L-Tyrosine 300 mg

L-Uracil 200 mg

Y-SD selection medium: Add H20 to 1 l

Yeast Nitrogen Base w/o aa. 6.7 g

Agar 20 g

Adjust to pH 5.8

Add H_20 to 850 ml and autoclave

Before use add:

40% dextrose 50 ml

10 x Dropout 100 ml

A single colony picked from Y-SD plates with L-Tryptophan and L-Leucine dropout medium (–TL plates) was inoculated in 5 ml Y-SD medium with L-leucine dropout (-L medium) overnight at 30°C. 100 µl of culture were used to inoculate 5 ml fresh –L medium, grown overnight and again 100 µl of the second culture was used to inoculate 5 ml YPD medium and grown overnight. 1 ml of the final culture was pelleted and lysed in lysis solution. The lysate was mixed with 1 volume phenol/chloroform and 100 mg washed glass beads in a DNA mixer for 5-10 min. The mixture was centrifuged and the supernatant was precipitated with 1/10 volume of

NaOAc and 1 volume of isopropanol. The pellet was briefly washed with 70% ethanol and dissolved in TE buffer.

2.10 Plant transformation

2.10.1 AP3 tag fusion constructs

***ap3-1*TDEF plants** were generated using a pBARA derivative where a 7kbp DEF genomic locus was cloned by EcoRI. The DEF genomic fragment was isolated by pTDEF (gift of Susanna Schwarz-Sommer).

CONT the promoter of *AP3* was isolated by PCR with primers AL193 and AL447, the coding sequence of AP3 was isolated by PCR with primers AL190 and AL191 and the CamV35S was isolated by PCR with primers AL190 and AL191. The promoter-coding sequence-terminator cassette was assembled in pBlueScript (Promega) cut with SacII and XhoI restriction enzymes, obtaining the pFCT construct. The complete cassette was excised from pFCT by XmaI and cloned into pBARA cut with XmaI.

All the following constructs were assembled in pFCT exploiting the artificial cloning site introduced either at the N or C terminus of AP3 coding sequence. The tag coding sequence was PCR amplified with the primers listed below. The cassette was then excised by XmaI and cloned into pBARA cut with XmaI.

TAPN AL210 AL207

TAPC AL208 AL209

3HN AL492 AL493

3HC AL494 AL495

3MN AL496 AL497

3MC AL498 AL499

6MN AL500 AL501

6MC AL502 AL503

GFPN AL504 AL505

GFPC AL445 AL446

All the N terminal fusions were inserted in pFCT by PstI restriction site (except GFPN that was PCR amplified and cut with NsiI and inserted in pFCT cut with PstI). All the C terminal fusions were inserted in pFCT by SalI restriction site.

2.10.2 Plant transformation in *Arabidopsis*

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

Infiltration medium

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

2.10.3 Generation of transgenic lines of Arabidopsis

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

2.11 Methods for *in situ* hybridization *in planta*

In situ hybridization were performed as described by Zachgo (2002).

2.12 Primer sequences

AL1003	AATGTCGACTGCAGCAAAGCATCCAAG
AL1004	AAAGAATTCATGGGAAGAGGAAAAATTGAG
AL1005	CCAGTCGACGAAACGCTCCTGAAG
AL1028	AAGAGCAGTAGTGGTAGTGGTTCG
AL1030	GATCTGAAGCTAAGAAGTGGAAACC
AL1077	CCACCATTCCATAAAAAGCCTTGTCC
AL1080	CAAACGCACGAGAAGATTGG
AL1081	ATCGGACATCAGTCCACACCAG
AL1081	ATCGGACATCAGTCCACACCAG
AL1083	CCCAGCAAAATAACACCTTGAG
AL1084	ATCACTCTGGTTTAGCACTCTAGC
AL1104	CCACCCGAAAGTTAAGAGACTAAGG
AL1105	CATTAATTCAAATTCTTTCCATCTAGGC
AL1109	GATCGATTTCCCTTTACCATGC
AL1110	AGCGTCAATATTCTTGAGTGCC
AL1121	GTCAATCTTAATTGTCCTCCCCCTTTGACC
AL1122	CCTTGTTATCTTTTGGTCTCTCCATTTCC
AL1123	AAGAATTGAGAACTCAAGCAACAGGCAGG
AL1124	AAATTTCAGACGACGATTCAGGCTTCACCG
AL1125	ATGCATGAATTTTGTAGCCCTTCTACCACG
AL1126	TATTTACCTCATGTTTGGGATCCCAGAGCC
AL1144	GCAAAGATCTCGTCTAAACAAACGTACAG
AL1145	ACCAAAACACTCGAAAAAGAGAGCCAGAGC
AL1148	AGCGAATGAAAGTGAAACGTCGATTGCTCC
AL1149	CTGAAATACAAAATGCTAACTGTCTAACCG
AL1150	CTAGAAGAAAAATAGGCCAAAAATCCC
AL1151	CGAGTTAATTATAGAGGAAAAAGAACGCAAGC
AL1152	TTGATAGTACTTAAACCCAATTTCACACGC
AL1153	CAGCTCTTGACAGTGACATATCACG
AL1154	GGTTGAAGTACTCAAGTCACAGTGAAAGG
AL1155	GTGGTTCTGTCTGTTATTGTTGCAAGC
AL1156	CCCCGCATTTTACATGTGTGGTTCAGC
AL1157	TTGGAAATTGTATTTGCTGAGAAATATTTAGCC
AL1158	CCCTACTATGTTACAGCTTTTGGTCTACG
AL1159	GATCACGTGAGAACGATATATCAGTTAGC
AL1190	ATGCTTAGAACTTCCTCGATTCATCTC
AL1191	GAGATATTGACACGTGGGAAATCAACTC
AL1194	CAATTAAGGGCTAATTGACTTTGATATACCG
AL1195	TTGAAAATTCGTAGAGCTTCCCAGTGC
AL1377	CCGGAATTCATGCAATACCAGACAGAATCGTG
AL1378	CGGGGATCCTCAGAGCCAGAGAGCGCCGGCGTC
AL1379	CCGGAATTCATGCAATACAAAACAGAAACTCGAGG
AL1380	CGGGGATCCTCAGAGCCAAAGAGCGCCAGCATC
AL1444	AAAGTCGACTTATGGCACCAGATAACGACCATTTC
AL1445	AAAGTCGACTTAATGGCGAGAATCGGATGAAGC
AL1446	AAAGTCGACAAATGTCTGACGACCAATTCCATCACC
AL1447	AAAGTCGACTCAATGGCGAGAAATAGAGGAAGC
AL1450	AAAGAATTCATGGCCGACAACGACGGAGCAGTG
AL1451	AAAGTCGACTCAACGTGGTTCGTGGTCGTCTTCC
AL1452	AAAGAATTCATGGAGATGATGAGCTCTTCTTCTTC
AL1453	AATGTCGACTCAAGTGTGTTCTCGTGGACGAGC
AL1459	GTTGTCGACTCAGAGGACTCGGAGCTGAGCAAG
AL1463	AAAGAATTCATGATGGATAGAGGAGAATGCTTG
AL1464	AAAGTCGACCTAGTTAACCTTAGGCGGATCAGCTC
AL1477	TAAGAATTCATGGATGGATCAGGCACAGGCAGTAG
AL1480	AAACTCGAGCTAATCTTGCTGATCCTCCTCATCACC

AL1487	AAAGAATTCATGCAAAAGCCAACATCAAGTATCTTAAATGTC
AL152	CCTCAGTGGGTGTAGCTGTAATTATCCC
AL190	TAACCGCGGACTGCAGAATATGGCGAGAGGGAAGATC
AL191	AGCCTCGAGTTAGTCGACTTCAAGAAGATGGAAGG
AL193	CCTCTCGCCATATTCCGCGGTCTTTGTTTAATC
AL207	TAAGCTTATCGTCCTGCAGAAGTGC
AL208	GGAGTCGACATGGAAAAGAGAAG
AL209	ACGGTCGACATCAAGCTTCAGGTTGACTTCC
AL210	CCCCTGCAGATAACTTCGTATAGC
AL255	AAAGAATTCATGGGTAGAGGAAAG
AL256	ACGGTCGACTCAATCGATGACCAAAG
AL257	ATAGAATTCATGGCGAGAGGGAAG
AL303	AAAGCGGCCGCAATGGGTAGAGGAAAG
AL303	AAAGCGGCCGCAATGGGTAGAGGAAAG
AL304	ACGAGATCTTCAATCGATGACCAAAGAC
AL428	GAGGAAGAAAAGAGGGGTGTGATG
AL447	GTGACCGCGGACCACCCGGGGAGTGCGTGTTGTTTGTGTAG
AL482	GAAATACACAGAAACCTGGTGC
AL483	GAGTAGGATGGTGATTAGTCG
AL797	GCAAAGCTTAGTTTTGCTGGCCGCATCTTC
AL802	AAAACTAGTCCAAGTTTTTAGCTTTGGTCC
AL803	GGAGAATTCGAAGTAAAGGGTCCACTTGAG
AL804	ATTACTAGTGAAACAACACTAACTGGTCGAAGC
AL805	AATGAATTCGTTGAAGAGATTTGGTGGAGAGG
AL827	AAATCATGATAATGTCGAAAGCTACATATAAGGAACGTGC
AL867	TAAAAGCTTGGTTATTACTGAGTAGTATTTATTTAAG
AL868	ATAAAGCTTGAATTCATGGCGAGAGGGAAG
AL893	ACCGTCGACTTACACCTTTCTCTTCTTCTTAGGTTCAAGAAGATGGAAGG
AL895	ACCAGATCTTTACACCTTTCTCTTCTTCTTAGGATCGATGACCAAAGAC
AL934	ATTGTCGACCTCTTTTTTTGGGTTTGGTGGGG
AL935	CCACTCGAGCCAAAAAAGAAGAGAAAGGTCG
AL939	ATAGAATTCATGGCTCGAGGGAAG
AL941	ACCGTCGACTTACTCAAGCAAAGCAAAAGTGG
AL943	AAAGCGGCCGCATGGGAAGAGGAAAAATTGAG
AL945	ACGAGATCTCTAGAAACGCTCCTGAAG
AL947	ATAGAATTCATGGGGAGAGGGAAAG
AL949	AAAACTAGTCTGACTCAAATTCATTTGCC
AL950	GGAGAATTCGGCATTTTTCTATTGTGGG
X16	CGGTGCTTTAAATGGGGGGACCCGATACCC
X22	CCAAAAGATAACAAGGTGCAACCACGG
X23	GGGCTACAAAATTCATGCATCTTGCCAGAGC
591	CGAACATGCCACAGCTAAATAAC
733	GCTTGAATAATTCTACCTGAGTACTGCAAG
740	AAATGTCAATCTTCTCTACAAACTTGCGC
748	GAAAGGCACACCATCTACTTAACTAATC
749	GTTTGGGATAATTACAGCTACACCCACTG
750	CGTACCCCCTCATGTTAGTAAAGATTTGC
751	GCTCTTTTAAAGCCACATAAACCGTAATACC
811	AACAAATCTATTTACCACCTCTCGATACCC

3. RESULTS

The development of the flower starts when a determinate floral meristem is produced from an indeterminate inflorescence meristem, that gives rise to the primordia of four different kinds of organs.

ROXY1 is a newly characterized gene of *Arabidopsis*, involved in the initiation of petal primordia, whereas class B homeotic genes control further organogenesis.

In the work presented here these two groups of genes, involved in petal initiation and development, are studied. In particular, it is investigated how they perform their tasks: target genes of class B transcription factors are investigated by Crosslinked Chromatin Immunoprecipitation (X-ChIP) experiments. Interactions of class B proteins among each other and with other factors are investigated taking advantage of the yeast system.

ROXY1 encodes for a glutaredoxin thought to be involved in posttranslational modifications of interaction partners, i.e. target proteins. A yeast two-hybrid screening was carried out to isolate target proteins of ROXY1, and selected interactors were further characterized.

3.1 Establishing the X-ChIP TECHNIQUE

3.1.1 X-ChIP technique overview

The X-ChIP technique is used to demonstrate an *in vivo* interaction between a protein and a DNA fragment. Other techniques have been used to prove the formation of DNA-protein complexes, in yeast, by the one-hybrid system, or *in vitro*, by gel retardation assays. However, to study the class B factors-DNA interaction, the X-ChIP technique was preferred because offers the opportunity to study *in vivo* protein-DNA interactions in an organ specific fashion.

Figure 5 schematizes the X-ChIP technique procedure: in the first step, the material under investigation is incubated with paraformaldehyde (PFA) that induces covalent bonds between cytosine and lysine and between lysine and lysine, fixing DNA to proteins and proteins to proteins. Since the interaction between the protein under investigation and the DNA occurs in the nucleus, nuclei are extracted from fixed tissues, and chromatin is sheared by sonication. The sonication step is required to break and solubilize the fixed chromatin, since the PFA-induced crosslinks prevent DNA shearing by biochemical treatments, such as enzymatic restrictions (Orlando et al., 1997). Under the chosen condition, sonication breaks the chromatin randomly, producing DNA fragments 400 to 500bp long on average.

In order to isolate DNA fragments bound to the protein under investigation, a specific antibody is applied to the sheared chromatin, and the antibody-protein-DNA complexes are precipitated. Finally, crosslinks are reverted and the DNA is recovered.

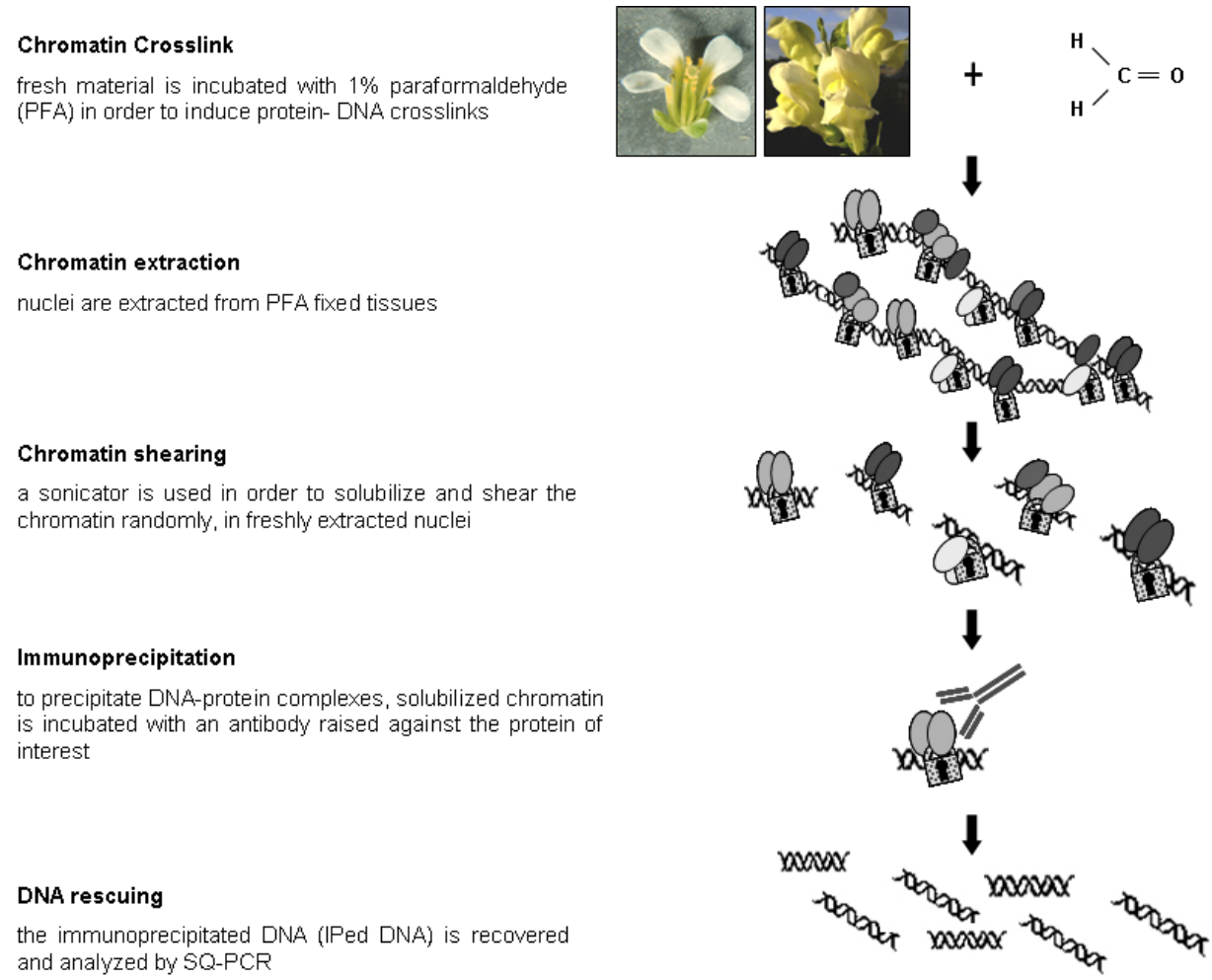


Figure 5. X-ChIP technique overview.

3.1.2 Immunoprecipitated DNA analysis

The IPed DNA, i.e. the pool of DNA isolated in an X-ChIP experiment, is represented by DNA fragments with a length between 200 and 1500bp; the largest portion is represented by 400-500bp long fragments. Due to the specific serum-protein interaction, the DNA fragments containing the protein binding element should be enriched with respect to other DNA species, that represent the background. Due to the random DNA shearing the immunoprecipitated fragments vary in their length. For this reason, in the IPed DNA pool, the concentration of DNA regions decreases proportionally with their distance from the binding site. Theoretically, it follows a Gaussian curve having as climax the most highly represented region, i.e. the 300bp surrounding the binding site. As the “distance from the binding site”/”DNA

concentration" relationship fits a Gaussian pattern only theoretically, in this work it will be referred to more generally as a bell shaped curve (Figure 6).

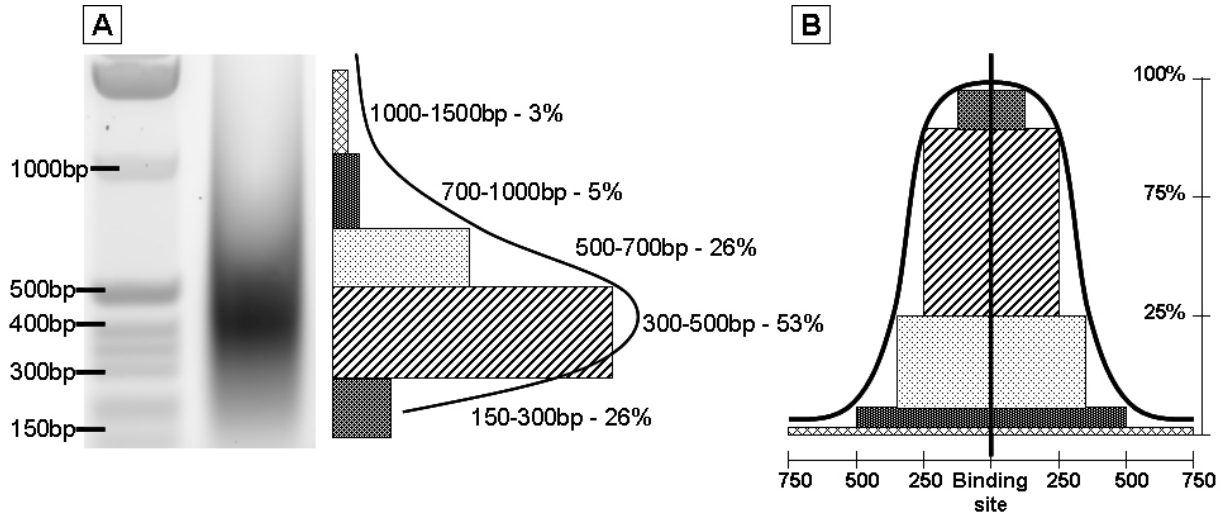


Figure 6. Distribution of sheared DNA according to its length, and the enrichment, in the pool of IPed DNA, of DNA areas according to their distance from the binding site.
A) DNA sheared by sonication was loaded on an EtBr/Agarose gel. The graph reports the relative abundance of DNA according to its length.
B) "distance from the binding site"/"specifically immunoprecipitated DNA" relationship follows a bell shaped curve. Due to the random shearing by sonication the most enriched sequences are not longer than 700bp. Regions further than 500bp away, represented only ~10% of the immunoprecipitated fragments, and the most represented region is a 300bp long area spanning the binding site. For simplification, the binding site was assumed to be in the center of the DNA fragment and the length interval was represented only by the longest fragment.

In order to asses whether a given DNA sequence contains an element bound by the protein under investigation, its enrichment is measured by Semi Quantitative Polymerase Chain Reaction (SQ-PCR), using specific primers. In the same way the enrichment of other areas, a few hundreds base pairs up- and downstream of the putative binding site, is also evaluated. The enrichment of these surrounding regions should decrease as their distance from the binding site increases, being the concentration of long DNA fragments inversely proportional to their size.

A "background control" is included: at least one primer pair is used for the amplification of a region further than 1000bp away from the binding site, that should not be pulled down by the immunoprecipitation. However it was chosen to be not further than 5000bp away from the binding site, as the chromatin density should be comparable. Thereby, the "background control" should represent DNA that was unspecifically immunoprecipitated and should therefore reveal the lowest value.

3.1.2.1 SemiQuantitative-PCR

In order to evaluate the relative enrichment of the putative binding site, of the surrounding regions and of the “background control”, SQ-PCRs are performed (Figure 7A and 7B). It is important to consider that the investigated regions are amplified with different primers and SQ-PCRs are performed on DNA immunoprecipitated from different tissues (wild type and mutant, see below), then normalization steps are required. The procedure, exemplarily shown in Figure 7, can be divided in four steps:

1) At least three SQ-PCR sets are carried out: using as template a) IPed DNA from wild type tissue b) IPed DNA from tissue were the protein under investigation is totally absent (e.g. a null mutant), and c) “Input” DNA. “Input” DNA is a sample aliquot taken after the DNA shearing, but before the immunoprecipitation step, that was then diluted down to a concentration similar to that of IPed DNA (see materials and methods).

2) The products of the three SQ-PCR sets are loaded on an EtBr/Agarose gel and band intensities are measured using a PhosphorImager scanner. The band intensities corresponding to the SQ-PCR sets a) and b) depend on the concentration of the DNA area that is being amplified and on the properties of the primers. The band intensities corresponding to the SQ-PCR set c) depend only on the properties of the primers, as the concentration of each DNA fragment is not affected by an immunoprecipitation step (Figure 7B). Values reported in Figure 7C show that band intensities of the wild type sample are generally higher than that of the mutant sample. This indicates that the total amount of IPed DNA from the wild type is more than that from the mutant.

3) The results of SQ-PCR sets a) or b) can be normalized according to their primer strength: the value of a) or b) bands is divided by the corresponding value of the c) band (wt/In and m/In respectively). The resulting value is the relative enrichment of the DNA sequences under investigation (Figure 7C).

4) Each value is divided by the average (avg) of its own set (wt or m) obtaining wt/wt-avg and m/m-avg values, respectively. This step is necessary to bring results from different experiments (i.e. replicates performed independently, on different tissues or organs) to the same order of magnitude.

The trend of the lines extrapolated from the latter values (point 4) can then be compared: if the protein under investigation actually binds the area considered, a bell

shaped curve, having the binding site as climax, should be extrapolated from the set of normalized data a) (as anticipated in the Figure 5). However a flat line from the mutant samples b) would indicate no antibody-protein-DNA binding activity in absence of the protein under investigation, confirming the specificity of the antibody-protein interaction (Figure 7D).

3.1.2.2 Statistical analysis

Due to an intrinsic high variability of the Polymerase Chain Reaction, each experiment was reproduced many times; the average and standard deviation of experiment results were calculated for every set of data, and statistical analysis was carried out.

The statistical analysis was conducted to demonstrate that a bell shaped curve or a flat line, could be extrapolated from data sets obtained from wild type or from mutant samples.

A bell shaped curve can be drawn because the set of data, generated using primers spanning the binding site, is significantly divergent with respect to the set of data corresponding to the background control.

A flat line is generated when the variation between data sets is not significant.

Toward this goal, each data set is compared to each other, and the probability that they are part of the same population is calculated by the t Student test.

Such a statistical analysis was applied to demonstrate the validity of the starting hypothesis, i.e. that the putative binding site is actually bound by the protein under investigation. No statistics were carried out when preliminary data do not encourage this hypothesis.

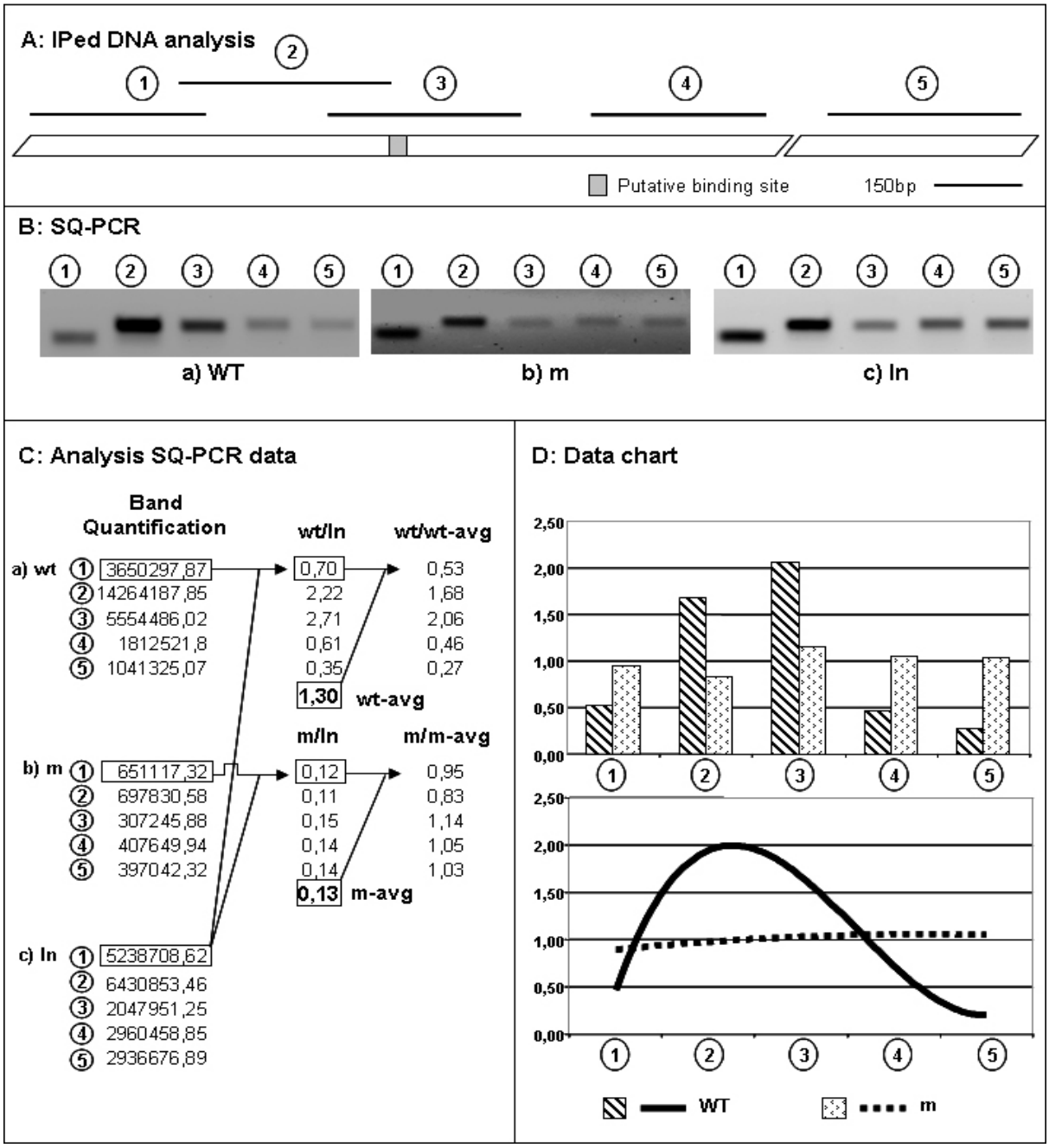


Figure 7. Strategy to quantify data produced in X-ChIP experiments
A) Schematic structure of a hypothetical DNA locus, where the studied protein is supposed to bind. Investigated regions are reported: regions 2 and 3 span the putative binding site (grey square) and regions 1 and 4 are 600bp away from it; region 5, the "background control", is about 1500bp away from the binding site.
B) SQ-PCRs are performed on a) "wild type" (wt) and b) "mutant" (m) IPed DNA, and on c) Input DNA (In).
C) Band intensities are quantified. Note that band intensities from the wild type sample (a) are generally higher than those from the mutant sample (b).
Values from wt or m are divided by the corresponding ones from In (wt/In and m/In). These values are divided by their average value (wt/wt-avg or m/m-avg).
D) The values wt/wt-avg and m/m-avg from panel C are plotted on the upper graph. The lower graph represents the trendlines extrapolated from the data: a bell shaped curve for wt, and a flat curve for mutant.

3.2 X-ChIP RESULTS

The X-ChIP technique was applied to study target genes of class B transcription factors in *Antirrhinum majus* and *Arabidopsis thaliana*. *Antirrhinum* is a good model system for the study of flower development, since large floral organs can be harvested separately already at relatively young stages. In the X-ChIP experiments reported below petals or stamens were harvested from stage 3 flowers, according to Bey et al. (2004), which have a size of 0,8-1 cm and are in an intensive growth phase. *Arabidopsis*, on the other hand, is the most commonly used plant model system: it is easy to be transformed, has a short life cycle, and its genome is completely sequenced (The Arabidopsis Genome Initiative, 2000; Somerville and Koornneef, 2002). However, due to the limited size of its flower, only the whole inflorescence can be collected for X-ChIP experiments, and not separate organs.

3.2.1 X-ChIP experiments in *Antirrhinum majus*

Class B transcription factors in A*ntirrhinum* are represented by DEF and GLO, two MADS box proteins that interact *in vitro* with DNA as heterodimers. The short DNA motif the DEF/GLO heterodimer binds, to regulate the expression of target genes, is called CArG box. The first genes that were postulated to be regulated by the DEF/GLO heterodimer are *DEFICIENS* and *GLOBOSA* themselves, which contain in their promoter one and three CArG boxes, respectively.

Two further genes, *DEFH125* and *EXTENSIN*, were indicated as possible target genes of DEF/GLO: the first is expressed in microspores and pollen, where *DEF* and *GLO* are not expressed (Zachgo et al., 1995; Zachgo et al. 1997), hence its expression could be repressed by the heterodimer; the second was isolated by Bey et al. (2004) being regulated by DEF/GLO.

Preliminary X-ChIP experiments conducted by Müller (2004) showed an enrichment of the promoter sequences of these four genes in samples extracted from wild type petals and immunoprecipitated with anti-DEF serum with respect to samples treated with pre-immune serum.

The *in vivo* interaction of DEF/GLO with the promoter of these four putative direct target genes (*DEF*, *GLO*, *EXTENSIN* and *DEFH125*) was tested with the above described X-ChIP procedure, allowing to compare normalized values.

3.2.1.1 DEF/GLO-G*LOBOSA* promoter interaction studies by X-ChIP experiments

In vitro assays demonstrated that the DEF/GLO heterodimer binds two of the three CArG boxes present in the *GLO* promoter. *In vivo* studies reported a failure of *GLO* transcription maintenance in *def* or *glo* mutants, suggesting that the interaction occurs *in vivo* to maintain the *GLO* expression (Tröbner et al*.,* 1992).

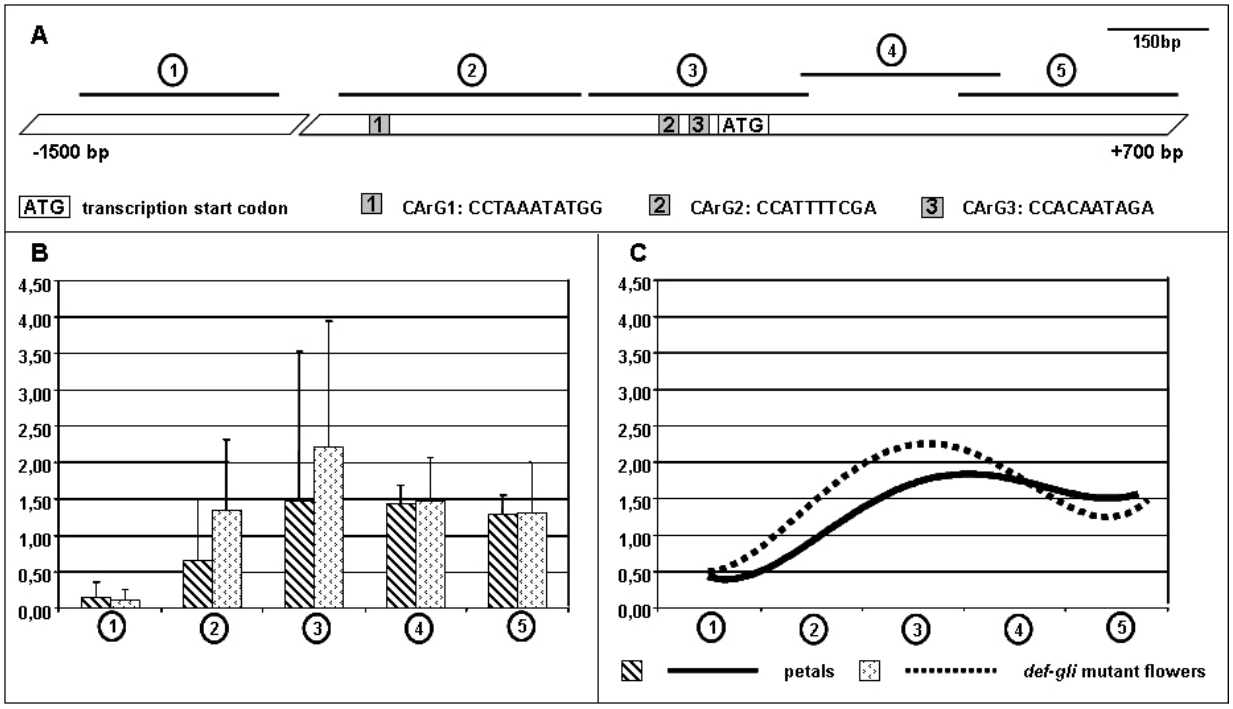


Figure 8. Investigation of the *GLOBOSA* genomic locus by X-ChIP
A) Structure of the *GLOBOSA* promoter with the three putative CArG boxes (CArG1, CArG2 and CArG3). DEF/GLO is able to bind CArG2 and CArG3 *in vitro*. Five areas were considered, spanning the putative binding sites (primer sets 2 and 3), adjacent regions (primer sets 4 and 5) and a further region, located 1200bp upstream, representing the background (primers set 1).
B) The data reported represent the average of six independent replicate of the same experiment. The standard deviation was calculated for each value.
C) Trendlines extrapolated from data shown in panel B.

The enrichment of DNA sequences included in the promoter of *GLOBOSA* has been analyzed in X-ChIP experiments with material extracted from wild type petals and *def-gli* mutant flowers.

As already discussed, the trend of a curve extrapolated from “petals” data should fit a bell shaped curve that should not be reproduced when “*def-gli* mutant flowers” data are considered. The latter, representing the negative control, should approximate a flat line.

The results from six independent experiment replicates, whose average and standard deviation are shown in Figure 8B and 8C, do not support the *in vitro* data: a bell shaped curve, having the region 3 as climax, can be drawn both in presence (petals) and in absence (*def-gli* mutant flowers) of the DEF protein, hence the interaction between the DEF/GLO heterodimer and the promoter of *GLOBOSA* could not be confirmed.

3.2.1.2 DEF/GLO- *DEFICIENS* promoter interaction studies by X-ChIP experiments

The promoter of *DEFICIENS* contains one putative CArG box, located about 1200bp upstream of the ATG start codon. In an analogous way to *GLOBOSA*, DEF/GLO was shown to bind this motif by *in vitro* studies to maintain the *DEF* expression.

In order to confirm that this interaction occurs *in vivo*, X-ChIP experiments were carried out, where the enrichment of the areas belonging to the genomic locus of *DEF* was compared between samples extracted from wild type petals and *def-gli* mutant flowers.

In Figure 9A the *DEF* genomic locus is schematized and the enrichment of areas tested by X-ChIP experiments is reported. These areas include the putative binding site (indicated as 1), the regions surrounding the start codon (2) and an area comprising the STOP codon (3). In the sample immunoprecipitated from petals the regions numbered 2 and 3, being distant from the binding site, should be much less represented compared to the region 1; on the contrary, they should be equally represented in the *def-gli* mutant flowers. The region upstream of the CArG box could not be investigated because its sequence is not known. The region between the CArG box and the start codon contains two inverted repeat elements that are most likely responsible for the failure to design primers able to amplify this region.

The X-ChIP experiment was carried out in six identical replicates both on wild type petals and on *def-gli* mutant flowers; the average results and the relative standard deviations are reported in Figure 9B. When material from petals is used, the area

containing the CArG box is, as expected, enriched with respect to the two background controls. However, "*def-gli* mutant flowers" data show a similar enrichment on the regions 1 and 2, while the region 3 is underrepresented.

Due to the impossibility of collecting data from the areas adjacent to the CArG box, it is impossible to demonstrate that the region 1 is the climax of a bell shaped curve, hence the DEF/GLO binding site. Furthermore the "*def-gli* mutant flowers" data do not fit a flat line, but the areas included in the promoter region (1 and 2) are indeed enriched with respect to the region 3.

These data are therefore insufficient to confirm the *in vivo* interaction of DEF/GLO with the promoter of *DEFICIENS*.

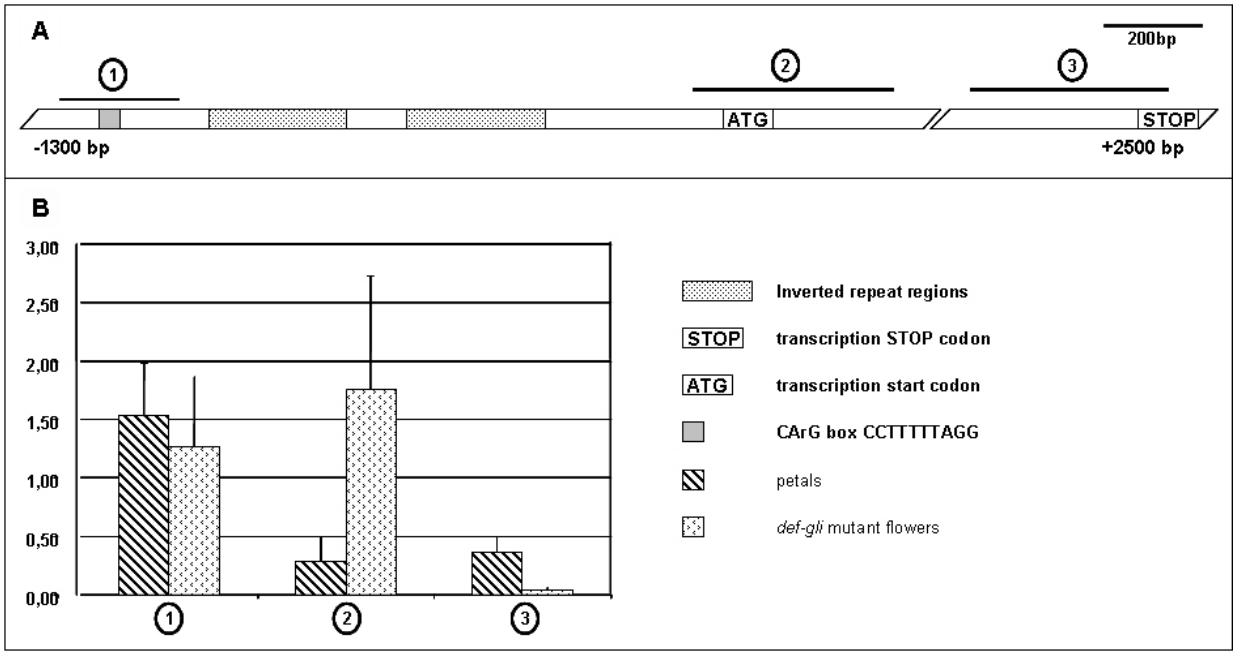


Figure 9. Investigation of the *DEFICIENS* genomic locus by X-ChIP
A) Structure of the *DEF* genomic locus with areas (1, 2 and 3) investigated in X-ChIP experiments.
B) Results of six independent replicas of similar experiments, carried out on petal and *def-gli* mutant flower material.

3.2.1.3 DEF/GLO-*DEFH125* promoter interaction studies by X-ChIP experiments

The *Antirrhinum majus* gene *DEFH125* codes for a MADS box transcription factor. *DEFH125* expression starts in young microspores that are formed after the meiotic division of the pollen mother cell, and continues during further pollen maturation. *DEFH125* is not expressed in any part of stamens or petals (Zachgo et al, 1997). As

already mentioned, the DEFICIENS protein is expressed at high level throughout the developing petals and stamens, but not in the pollen (Figure 10).

The complementary expression pattern of *DEF* and *DEFH125* raised the question if DEF/GLO could bind the promoter of *DEFH125* and thus might be involved in establishing the pollen specific *DEFH125* expression.

In order to validate this hypothesis the relative enrichment of five regions of the *DEFH125* promoter was compared by X-ChIP experiments conducted on material extracted from petals (six replicas), stamens (two replicas) and *def-gli* mutant flowers (six replicas). These regions span three putative CArG boxes, adjacent areas and an area located 2000bp upstream of the start codon, that served as background control (Figure 11A).

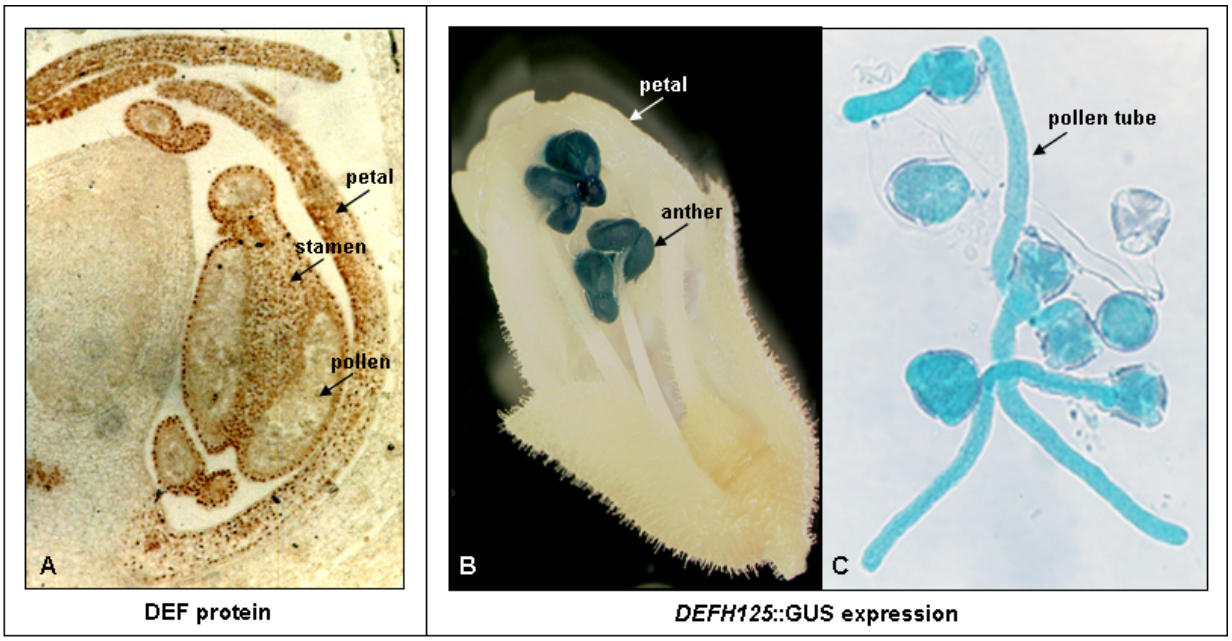


Figure 10 DEF and *DEFH125* expression analysis.
The *in situ* immunolocalization of DEF protein (A) is compared with the GUS reporter gene expression driven by the *DEFH125* promoter (B and C). DEF is expressed throughout petals and stamens, but not in developing pollen, where *DEFH125* mRNA is strongly expressed (courtesy of Sabine Zachgo).

The average values of the data collected from the X-ChIP experiments are reported in Figure 11B, together with the corresponding standard deviation. Considering the trendlines extrapolated from these data, it appears clear that they resemble a bell shaped curve in the case of “petals” and “stamens”, and an almost flat line in “*def-gli* mutant flowers”.

In order to corroborate these considerations it was investigated whether the differences between data sets were statistically relevant, by performing a t student

test. The numbers reported indicate the probability that the two compared sets of data are actually from the same population (Figure 11C).

Setting the significance threshold at 5% (Draghici, 2003), one can claim that the differences registered for all the "*def-gli* mutant" values can be attributed to statistical variance.

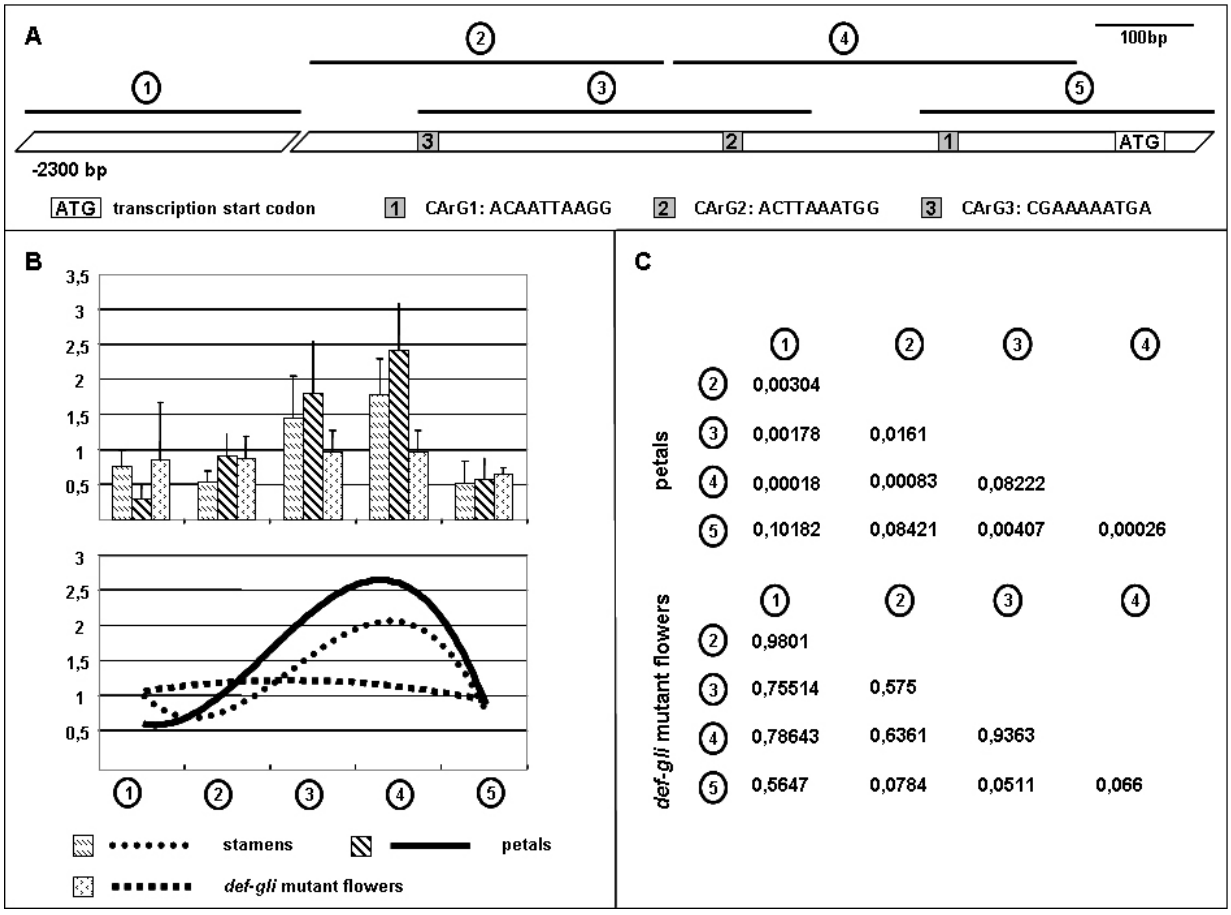


Figure 11. Investigation of *DEFH125* genomic locus by X-ChIP
A) Structure of the *DEFH125* promoter. Three putative CArG boxes are reported. Five sets of primers were designed to investigate the first 800bp of the promoter and a region representing the background, located 2000bp upstream of the translation start codon.
B) Average of the data collected for "stamens", "petals" and "*def-gli* mutant" X-ChIP experiments are plotted together with the relative standard deviation. Trendlines extrapolated from these data are reported in the lower panel.
C) A t student test was performed to investigate whether the differences between data were statistically relevant. The numbers reported indicate the probability that the two sets of data compared are actually from the same population.

On the other hand, this statistical analysis excludes that in "petals" the differences between the region 3 or 4 and the "background control" 1 can be due to sampling bias. Although the average value of samples 4 is higher than samples 3, there is 84% probability that this difference is imputable to statistical variance. The conclusion

would be that DEF/GLO actually interacts with the *DEFH125* promoter on a site located on the overlapping part of regions 3 and 4, that most likely coincides with the CArG box 2.

A complete statistical analysis will be conducted to confirm the “stamens” data.

3.2.1.4 DEF/GLO-*EXTENSIN* promoter interaction studies by X-ChIP experiments

The transcription of an *EXTENSIN*-like (*EXTENSIN*) gene was shown to be positively regulated by DEF/GLO, and to have a role in cell extension (Bey et al., 2004).

An X-ChIP experiment was carried out with the intention of investigating whether DEF/GLO binds the *EXTENSIN* locus *in vivo*. For this purpose, primers were designed to investigate six areas located in the *EXTENSIN* promoter, intron and coding sequence close to the STOP codon (Figure 12A).

The result data, reported in Figure 11B, show that none of the primer pairs span an area particularly enriched and that the variation in both samples is very limited. Therefore, these data do not support a direct interaction of DEF/GLO with the investigated 4800bp long *EXTENSIN* genomic locus.

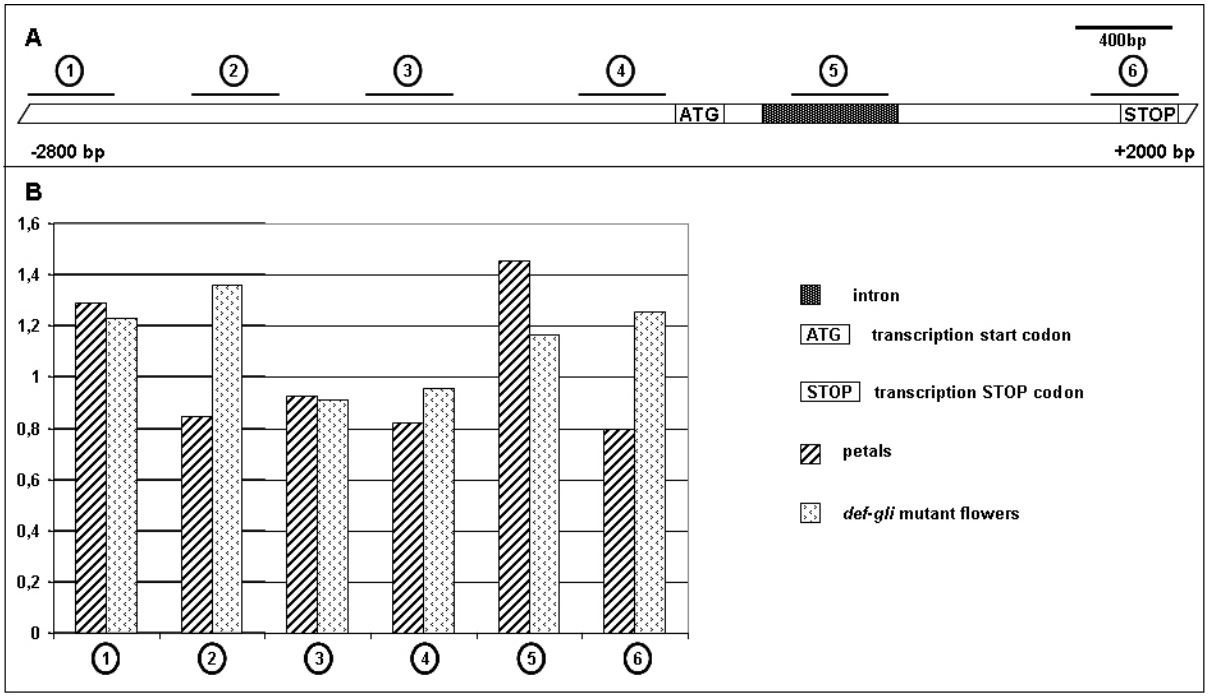


Figure 12. Investigation of *EXTENSIN* genomic locus by X-ChIP
A) Structure of the *EXTENSIN* genomic locus. Six sets of primers were designed to scan the entire region for enrichments. B) Representation of data collected from petals and *def-gli* mutant flowers: no area is particularly enriched.

3.2.1.5 Isolation of unknown targets of DEF/GLO by X-ChIP experiments

The isolation of new target sequences by X-ChIP has been reported in the literature. For instance Nelson et al. (2004) showed, with an experiment performed on human cell lines, that the background could be reduced by a tandem double immunoprecipitation. The recovered DNA, that should contain more target sequences than the DNA isolated with a single immunoprecipitation, was cloned and sequenced. A similar experiment was carried out with material extracted from wild type petals. The sheared chromatin was treated with two successive immunoprecipitation steps using an anti-DEF and anti-GLO polyclonal serum.

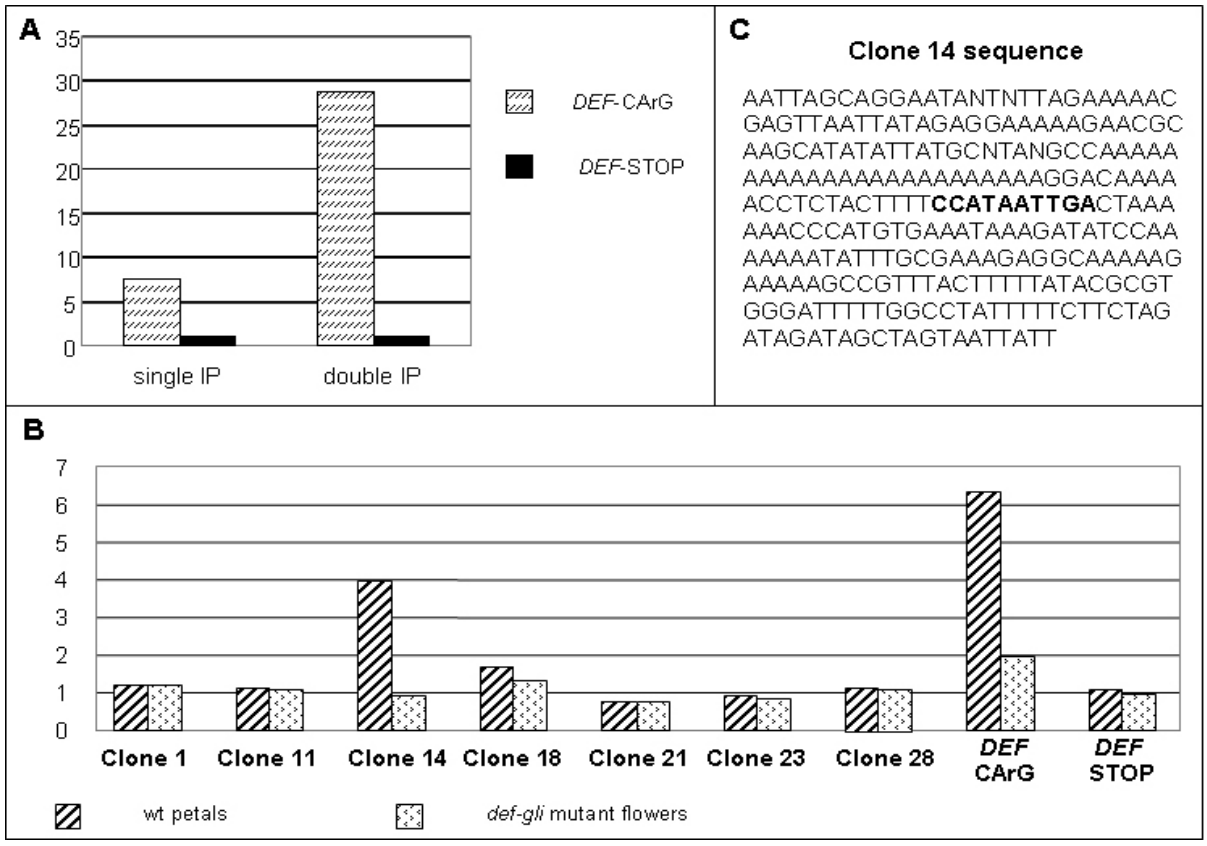


Figure 13. Isolation of unknown sequences bound by DEF/GLO
A) The ratio DEF-CArG/DEF-STOP representing specifically immunoprecipitated DNA vs. background increased after the second immunoprecipitation step, carried out with an anti-DEF and anti-GLO serum.
B) The relative enrichment of seven sequences, isolated by double-immunoprecipitation, was tested independently with specific primers, in an X-ChIP experiment, where DEF CArG and DEF STOP were also included as positive and background control, respectively. The experiment was performed on petals and *def-gli* mutant flowers. Clone 14 was significantly enriched.
C) Nucleotidic sequence of clone 14. A putative CArG box is highlighted.

The relative enrichment of the *DEF* promoter CArG region (*DEF*-CArG, region 1 in Figure 9) with respect to the background control (*DEF*-STOP, region 3 in Figure 9) was measured on IPed DNA rescued from a single immunoprecipitation using the anti-DEF serum, and from a double one, using the anti-DEF and anti-GLO serum. The ratio *DEF*-CArG/*DEF*-STOP increased almost 5 fold, from 6 in the single immunoprecipitation to 28 in the double immunoprecipitation experiment (Figure 13A).

The rescued DNA from this double immunoprecipitation was ligated with the pGEM-T vector, and 7 randomly picked clones were sequenced. To evaluate the relative enrichment of these sequences with respect to one to each other and to *DEF*-CArG and *DEF*-STOP, specific primers for these 7 sequences were designed and used in new and independent X-ChIP experiments.

One sequence, named clone 14, was significantly enriched with respect to *DEF*-STOP, considered as background, and contained a putative CArG box.

The flanking sequences of clone 14 were isolated and sequenced for 1500bp upstream and 1500bp downstream. No coding region was found, and the DNA sequence does not show any significant similarity to any gene in the database, although the investigated region might be too short to identify an ORF. However, investigating the clone 14 and adjacent sequences in future X-ChIP experiments, could allow to draw a bell shaped curve, hence confirming the interaction between clone 14 and DEF/GLO and locating the binding site corresponding to the climax.

3.2.3 X-ChIP experiments in *Arabidopsis thaliana*

Using *Arabidopsis thaliana* as model system offers many advantages in comparison to *Antirrhinum* (Somerville and Koornneef, 2002). For instance, accessibility to full genomic sequence allows investigating by X-ChIP experiments any area of interest without time-consuming isolation and sequencing of genomic DNA fragments.

AP3 and PI MADS box transcription factors represent the orthologs of DEF and GLO, respectively. Similarly to the *def* mutant in *Antirrhinum*, a mutation in *AP3* causes a homeotic conversion of petals into sepals and stamens are transformed into carpels. Furthermore AP3 forms a heterodimer with PI to bind CArG boxes in genomic DNA (Hill et al., 1998).

A few genes have been postulated to be regulated directly by the AP3/PI heterodimer, among these there are *AP3* and *NAP*. The expression of *AP3* is reduced in *ap3* or *pi* mutants, suggesting that AP3 and PI are required to maintain *AP3* expression in a positive feedback loop. *In vitro* experiments demonstrated that the AP3/PI heterodimer can bind two of the three CArG boxes contained in the *AP3* promoter, suggesting that the heterodimer acts directly on the *AP3* promoter.

NAC-LIKE, ACTIVATED BY AP3/PI (*NAP*) is an *Arabidopsis* gene that was proposed to be a direct target of AP3/PI, because its transcription was upregulated in an AP3 inducible system, although the interaction AP3/PI-*NAP* genomic locus was not proved (Sablowski et al. 1998). *NAP* is expressed relatively late in floral development, in developing petals and stamens, and is thought to play a role in the transition from the cell division to the cell expansion phase of organ growth.

In this work, the easy and rapid transformability of *Arabidopsis* has been exploited to create two kinds of transgenic plants: in the first, the entire *DEF* genomic locus was introduced into *ap3-1* mutant plants (*ap3-1*TDEF, see 3.2.3.1), and in the second the same mutant was complemented with constructs expressing AP3 fused to different peptide tags (see 3.2.3.2). Transgenic inflorescences were used to carry out X-ChIP experiments with the same anti-DEF serum employed with *Antirrhinum* in the first case, and with commercially available monoclonal antibodies raised against the tag fused to AP3 in the second case.

The *ap3* mutant background was chosen for two reasons: firstly to test the capacity of DEF to replace AP3 and the functionality of the chimeric proteins, by mutant phenotype complementation, secondly to avoid competition in target sequence binding between the transgenic and the endogenous AP3.

Among many alleles of *ap3, ap3-1* represents a weak and temperature sensitive allele that shows a wild type-like phenotype at 15°C and a mutated phenotype at 26°C (Bowman et al*.,* 1988; Sablowski et al., 1998). The advantage of using this line is that the homozygote is fertile at 15°C and can be easily transformed, thus circumventing further time-consuming selfing steps.

3.2.3.1 DEFICIENS can replace AP3 in the *ap3-1* TDEF system

The 7kbp *DEF* genomic locus (provided by Dr. Z. Schwarz-Sommer) was cloned into the binary vector pBARA, obtaining the construct pTDEF, and introduced via *Agrobacterium tumefaciens* mediated transformation into *ap3-1* mutant plants. 54 transgenic lines were obtained; the majority of them showed a rescued phenotype at 26°C, in accordance with what has been reported in the literature (Samach et al., 1997).

Generally, petals were white with occasional green sectors, and variation between stamenoids and stamens were found in the third whorl. Furthermore, as schematized in Figure 14A, the expression of the transgene *DEF* in the fourth whorl, where also endogenous *PI* is expressed at early stages (but not *AP3*), is the cause of *PI* expression maintenance. This leads to the formation of occasional additional stamens in the center of the flower, that reveal carpelloid features like stigmatic papillae (Figure 14D). These plants were named *ap3-1*TDEF (Figure 14). The best complemented lines were selfed and segregating populations were obtained; the phenotypes were ranging from *ap3* mutant to those described above.

Since DEF can substitute AP3, this reveals that the two proteins act in a similar way in a heterologous system, regulating a set of common target genes. Taking advantage of this, X-ChIP experiments were carried out using an anti-DEF serum and flowers harvested from *ap3-1*TDEF second generation plants that showed a good complementation grade. *NAP* and *AP3* genomic regions were investigated and results are summarized below, in Figure 17.

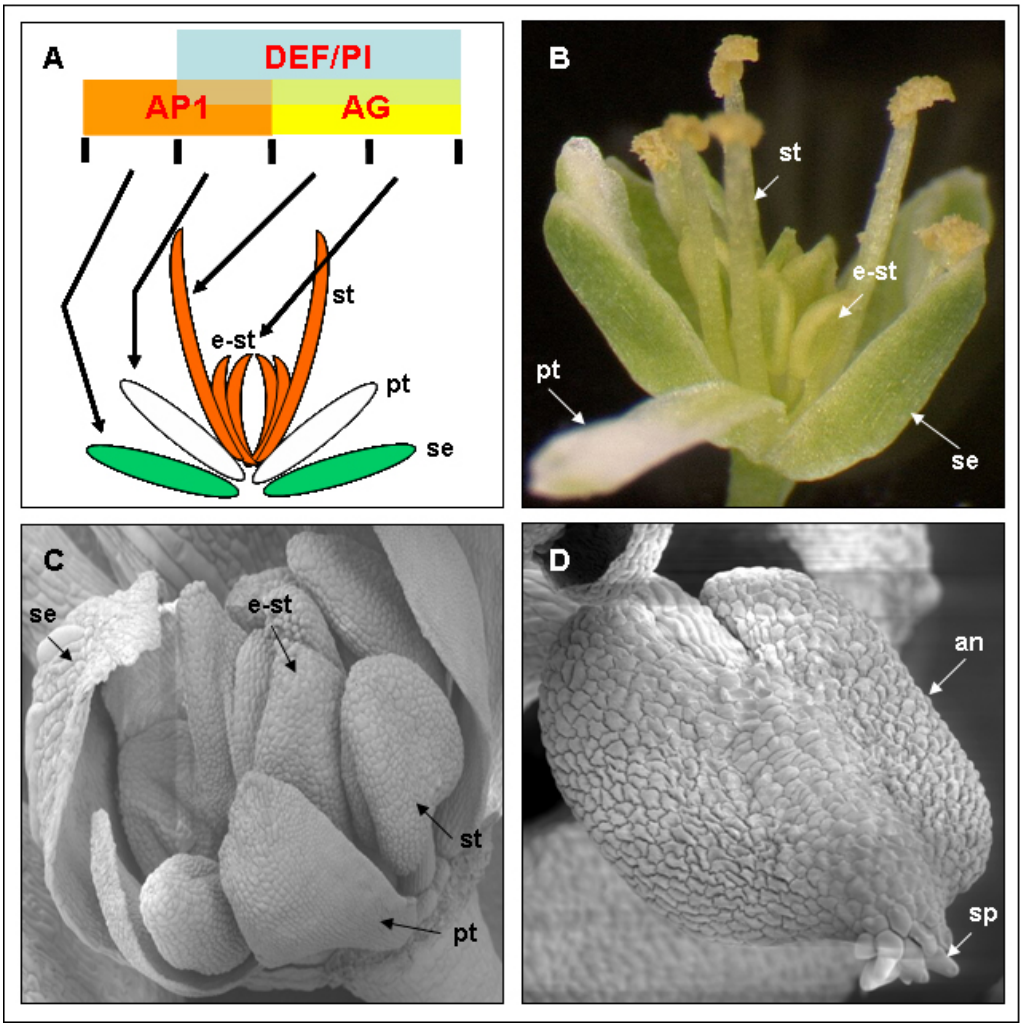


Figure 14. *ap3-1*TDEF plants grown at 26°C.
A) The overlapping expression of *DEF* and *PI* in the center of the flower is the cause of occasional ectopic stamen development. B) Complemented *ap3-1*TDEF flowers. Petals are almost completely white and an additional whorl of stamens develops in the center of the flowers. C) Scanning Electron Microscope (SEM) picture of an *ap3-1*TDEF flower at stage 12. D) SEM picture of an anther carrying feminized traits.
se sepals, **pt** petals, **st** stamens, **e-st** ectopic stamens, **ant** anthers, **sp** stigmatic papillae.

3.2.3.2 Production of tagged AP3 proteins

The full length *AP3* coding sequence was cloned downstream of 1200bp from its own promoter, that was shown to be sufficient to drive an *AP3* wild type-like expression (Hill et al., 1998; Jenik et al., 2001), and upstream of the Cauliflower Mosaic Virus (CaMV) transcription terminator. A selection of DNA fragments coding for various peptide tags was cloned at the 3' and 5' end of the *AP3* coding sequence. The peptide tag selection comprised a three times repetition of influenza A virus haemagglutinin (3HA), a three times repetition of human c-MYC tag (3MYC), a six

times repetition of human c-MYC tag (6MYC), the green fluorescent protein (GFP) and the Tandem Affinity Purification tag (TAP, Puig et al., 2001) (Figure 15).

These eleven constructs were prepared to test the ability of the tagged versions of AP3 to replace the endogenous AP3 in *ap3-1* mutant plants, which would allow to use commercial antibodies raised against the tag to perform X-ChIP experiments.

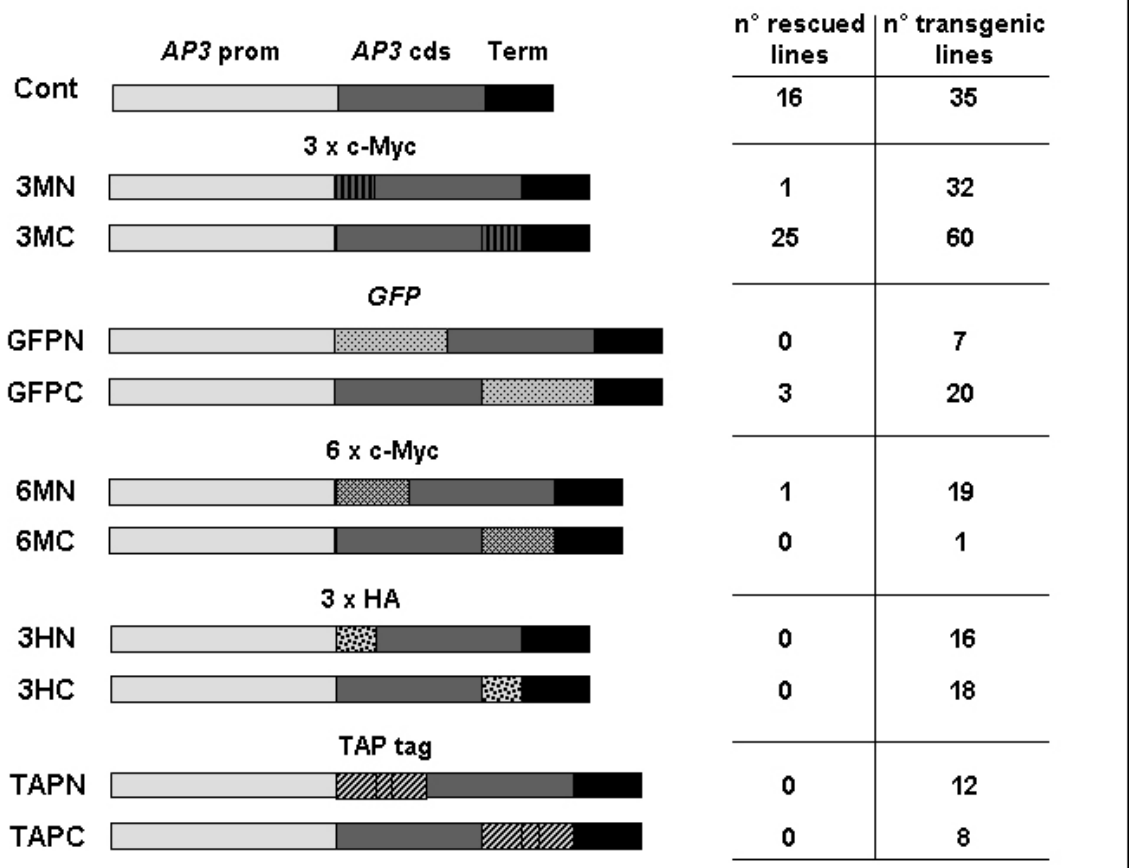
The selection of tags was made with the intent of employing small peptides (3HA, 3MYC or 6MYC) that are most likely not interfering with the AP3 activity, but against whom only monoclonal antibodies are available. Large tags, like GFP, could be immunoprecipitated with polyclonal sera, which offer a higher interaction chance, although they might interfere more severely with the AP3 protein function.

The TAP tag is composed of two parts (Protein A and Calmodulin binding protein) separated by a TEV cleavage site. The TAP purification steps can be performed in a gentle native manner, that allows also to recover protein complexes that include the tagged protein and to analyze them by mass spectrometry. In this work it is addressed the question if the TAP procedure could be used in X-ChIP experiments.

As the tag position can have an impact on the normal protein activity, they were fused to both the C- and N- terminus of AP3, to increase the chance of identifying a line expressing a functional fusion protein.

ap3-1 plants, as discussed, show a wild type-like phenotype at 15°C and resemble null mutant at 26°C. The eleven constructs reported in Figure 15 were introduced into *ap3-1* plants; transformants were grown at 26°C and screened for complemented wild type-like flowers. No complemented plant was observed for GFPN, 3HN, 3HC, 6MC, TAPN and TAPC lines. 3 GFPC plants, 1 6MN, 1 3MN and 25 3MC showed wild type-like flowers, although the rescuing of second and third whorl floral organs was only partial. Petals were shorter than in wild type flowers and showed large green sectors. Stamens had a short filament surmounted by an elongated and enlarged anther, and were totally sterile at 26°C (Figures 16D, 16E and 16F).

The complementation grade was higher with C-fusion constructs than with N-fusions (like GFPC and 3MC vs. GFPN and 3MN), suggesting that the impact of the artificial peptide on the AP3 functionality depends on its position.



	n° rescued lines	n° transgenic lines
Cont	16	35
3MN	1	32
3MC	25	60
GFPN	0	7
GFPC	3	20
6MN	1	19
6MC	0	1
3HN	0	16
3HC	0	18
TAPN	0	12
TAPC	0	8

Figure 15. Schematic representation of AP3 tagged constructs. The right table reports the number of transgenic lines per each construct (n° transgenic lines) and the number of plants that showed a wild type-like phenotype in the first generation (n° rescued lines). ***AP3* prom**, *AP3* promoter; ***AP3* cds** *AP3* coding sequence.

The wild type-like plants from 3MN, 3MC, 6MN and GFPC lines were selfed at 15°C, obtaining a second generation. The best complemented plants from all the second generation lines showed a better complementation grade than the wild type-like first generation lines. In the second generation plants, petals were larger and stamens showed longer filaments and smaller anthers, occasionally carrying fertile pollen (Figures 16B and 16C). This improvement could be related to an increased copy number of the transgene. Material for X-ChIP experiments was harvested from the best complemented individuals of the 6MN and GFPC second generation lines.

Figure 16. *ap3-1* mutant complementation.
ap3-1 flowers from plant grown at 26°C (G), and 15°C (A). (B) and (C) show wild-type like flowers from second generation 6MN and GFPC, grown at 26°C. (D), (E) and (F) report examples of wild type-like flowers from first generation 3MN, 3MC and GFPC grown at 26°C. (H) and (I) show flowers from first generation of 3HN and GFPN lines grown at 26°C, as representatives of transgenic plants where the mutant phenotype was not rescued.

3.2.3.3 *ap3-1*TDEF and tagged AP3 X-ChIP experiments

X-ChIP experiments were performed using inflorescences from the best complemented second generation plants from *ap3-1*TDEF, GFPC and 6MN lines. In the case of *ap3-1*TDEF the same polyclonal anti-DEF serum, employed for the *Antirrhinum* experiments, was used.

Commercially available monoclonal antibodies against the c-MYC tag, for 6MN, and a polyclonal serum against GFP, for GFPC, were used. Control experiments were carried out applying the same serum on material extracted from non transgenic *ap3-1* inflorescences.

NAP and *AP3* genomic regions were investigated. For *NAP* three primer sets were designed in the promoter, one in the first intron where there is a putative CArG box, and another one, representing the “background control”, located 5kbp upstream of the translation start point. For *AP3*, the 300bp upstream of the translation start codon, containing the putative binding sites, and a 1300bp downstream region, representing the background, were considered (Figure 17A).

Average values of three replicas of X-ChIP experiments carried out on *ap3-1*TDEF and *ap3-1* flowers (the latter used as control) were plotted on a chart: a bell shaped curve can be extrapolated from both sets of data obtained with primers spanning *NAP* regions (Figure 17B). Similarly to what has been shown for the *GLO* promoter (see 3.2.1.1), no conclusion can be drawn from this experiment. Although the *AP3* locus was not extensively investigated, the enrichment of the region where AP3/PI is supposed to bind, with respect to the background control (region 7, Figure 17) seems the same in both samples.

The output of X-ChIP experiments using anti-GFP serum on GFPC and *ap3-1* flowers (three replicates), and using anti-c-MYC on 6MN and *ap3-1* flowers (one replicate) showed no significant enrichment for any of the investigated areas.

The *Arabidopsis* experiments failed to bring convincing evidence for the interaction of AP3 or DEF with the regions under investigation. For *ap3-1*TDEF this failure could be explained with a cross reaction of the used serum with proteins different from DEF. For GFPC and 6MN the high background could prevent the detection of an AP3-DNA interaction. Possible explanations will be considered in the Discussion chapter.

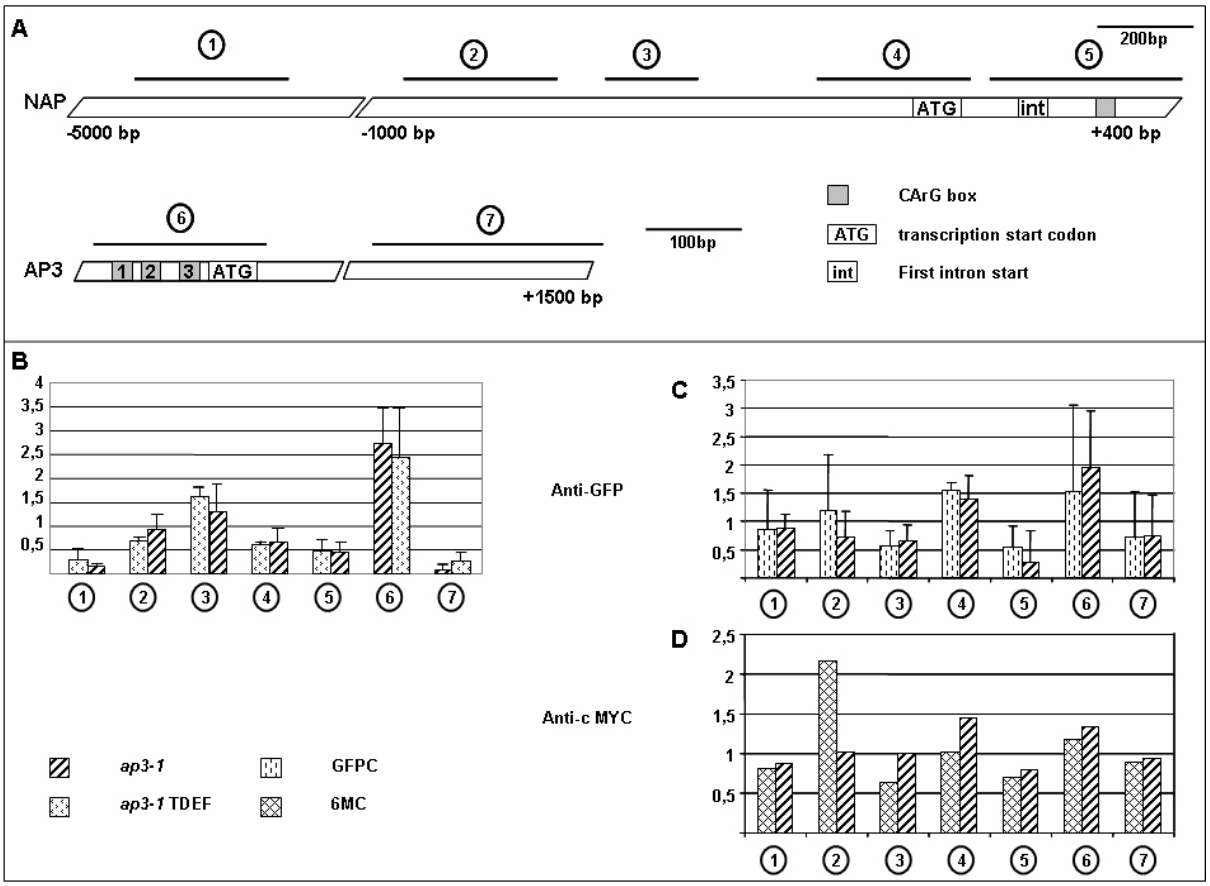


Figure 17. X-ChIP experiments on *ap3-1*TDEF, 6MN and GFP transgenic plants.
A) Structure of *NAP* and *AP3* genomic loci and primers sets spanning the investigating areas.
B) Average values of three replicates of X-ChIP experiments carried out on *ap3-1*TDEF.
C) Output of X-ChIP experiments carried out on GFPC and D) on 6MN systems.

3.3 *IN YEAST* AP3 INTERACTION STUDIES

Class B factors are able to promote the development of very different organs such as petals and stamens, that require the regulation of different target genes. Furthermore, the DNA-binding specificity of AP3/PI is very similar to that of other MADS box dimers (Riechmann et al*.,* 1996). These observations support the role of additional transcription factors participating in the specific protein-DNA complexes and thereby allowing a whorl specific activation or repression of target genes.

The interaction of AP3/PI and DEF/GLO with other MADS box factors has been described. SQUA enhances the binding activity of DEF/GLO, and AP1 and SEP3 form ternary complexes with AP3/PI and participate in the activation of target genes *in planta* (Egea-Cortines et al., 1999; Honma et al, 2001; Theißen and Saedler, 2001).

In order to isolate new partners of AP3/PI a three-hybrid screening (Figure 18) was carried out using AP3 and PI as baits. Furthermore, the ability of AP3/PI, DEF/GLO and partners to interact with each other and simultaneously with DNA in a way similar to that postulated by Theißen and Saedler (2001) and Egea-Cortines et al. (1999) (schematized in Figure 3) was tested in a new yeast system.

3.3.1 One – two - and three-hybrid systems

Due to its easy transformation and rapid growth, the eukaryotic organism yeast can be exploited to study DNA-protein interactions, using the one-hybrid system, and protein-protein interactions, by the two- and three-hybrid systems (Figure 18).

The yeast one-hybrid system is useful for investigating DNA-protein interactions: a DNA fragment of interest (bait) is cloned upstream of a reporter gene, and the GAL4 Activation Domain (AD), able to promote the transcription of the latter, is fused to a protein (prey). The transcription of the reporter gene occurs upon protein-DNA interaction (Fashena et al., 2000).

The yeast two-hybrid system is used to identify protein-protein interactions (Stanley Fields and Ok-kyu Song, 1989). A first protein (bait) is expressed as a fusion to the GAL4 Binding Domain (GAL4 BD), whose DNA binding element (UAS) is cloned upstream of a reporter gene. A second protein (prey) is expressed as a fusion to the

GAL4 Activation Domain (GAL4 AD). The interaction between bait and prey triggers the transcription of the reporter gene.

The yeast three-hybrid is a variant of the two-hybrid system: a third protein (TP), which could mediate or enhance the bait-prey interaction, is also expressed. This system can be exploited for the identification of interactions that require a third partner (Tanoue et al., 1999; Kawachi et al., 2001).

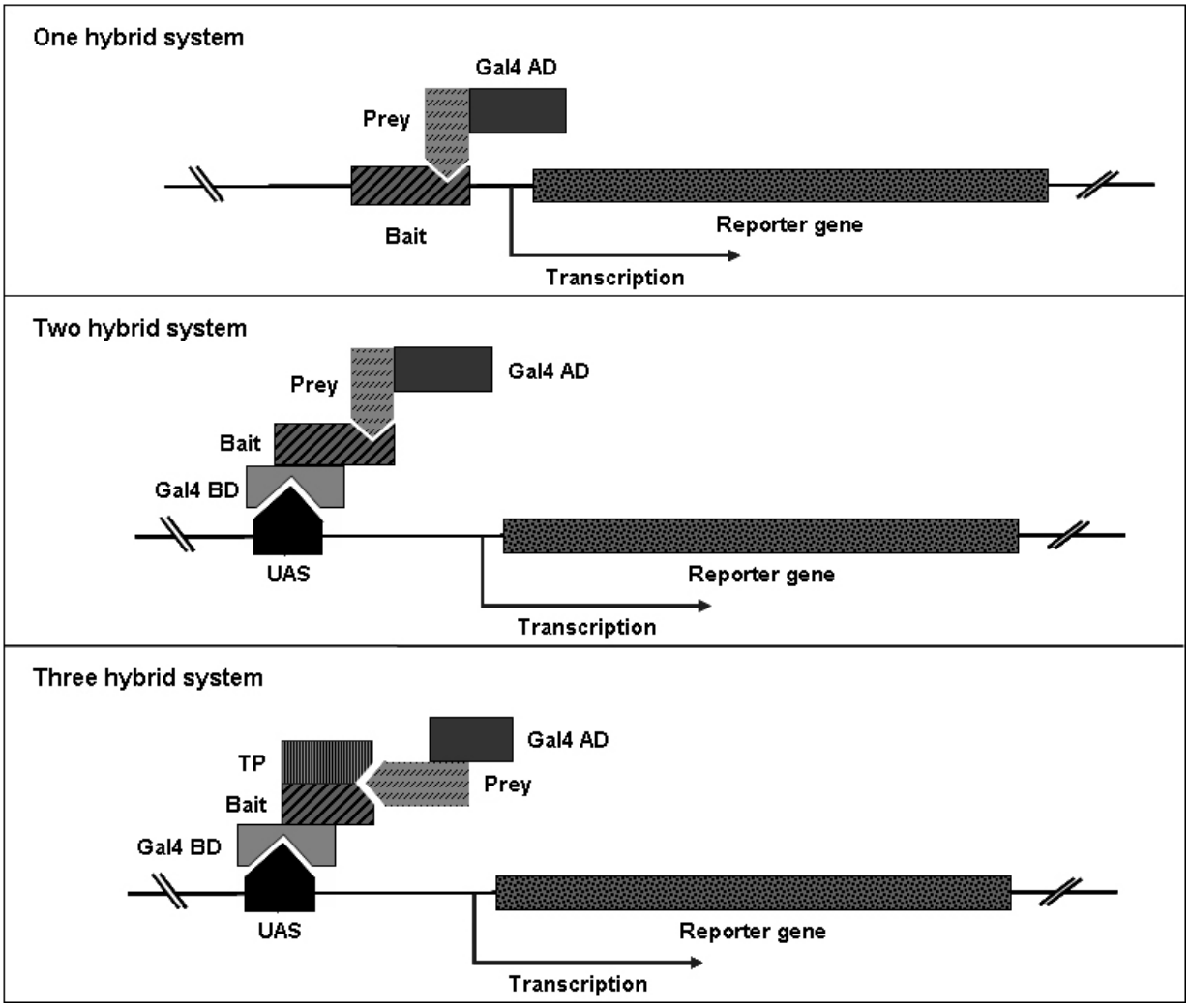


Figure 18. Yeast one- two- and three-hybrid systems.
A) Yeast one-hybrid system, useful for investigating the DNA-protein interactions. B) Yeast two-hybrid system, used to study protein-protein interactions. C) The yeast three-hybrid system, a variant of the two-hybrid system, can be exploited for the identification of interactions that require a third partner.

In the three systems, the prey protein fused to the GAL4 AD could be encoded by a cDNA library, thus allowing to identify new interaction partners. The reporter gene encodes an enzyme (such as HIS3 or ADE2) that allows the yeast strain (defective in this enzyme) to grow on selective media lacking certain amino acids (histidine and

adenine). Another kind of reporter gene encodes enzymes (such as LacZ) whose activity can be detected with an assay. The yeast strain used in this work carries both types of reporter genes.

3.3.2 The three-hybrid system is used to identify AP3/PI interactors

The three-hybrid system represents an extended version of the two-hybrid system and can be used either for library screenings, to identify new ternary interaction partners, or for testing the interaction of three putative complex members (Figure 18).
The p3HS3 vector is a pBRIDGE derivative and was prepared to express AP3 and PI simultaneously, the first cloned in frame with the GAL4 Binding Domain (GAL4 BD), and the second with a Nuclear Localization Signal (NLS), to ensure its translocation into the nucleus.
p3HS3 was first introduced into the yeast strain AH109 for an auto-activation test. The test gave negative results, since the yeast was unable to grow on YSD media lacking tryptophan (TRP, the pBRIDGE selective marker), histidine and adenine (HIS and ADE, amino acids corresponding to the reporter genes).
p3HS3 was then introduced into the yeast strain Y187 (MAT-α), that was subsequently mated to AH109 (MAT-a) containing an *Arabidopsis* whole plant library (provided by Dr. Simona Masiero and Dr. Hans Sommer) cloned in the pGADT7-Rec vector.
Diploids, obtained by the mating, were plated on media lacking TRP, leucine (LEU, pGADT7-Rec selective marker), ADE, HIS and 3-Aminotriazole (3AT) at the concentration of 1mM and grown at 22°C. 3AT is an inhibitor of HIS3, and is used to eliminate the background growth on media lacking HIS.
50 colonies, able to grow on selective media, were isolated and replated on fresh selective media containing 2mM 3AT and grown at 22°C and 28°C. A replica of this plate was used to perform a β-Galactosidase assay, to test the expression of the lacZ reporter gene. The library inserts of the survivors of the second selection step, and showing also GUS staining, were PCR isolated and sequenced. No differences were registered between the two different growth conditions, at 22°C and 28°C, although Honma et al. (2001) indicated 22°C as the optimal temperature for *Arabidopsis* MADS box protein interactions.

Many sequences were redundant, coding for the same gene. Only one clone per gene was considered for further characterization. The corresponding vector, containing the library insert, was isolated from the yeast colony and retransformed into AH109 in combination with either p3HS3 (to reconfirm the interaction), or p3HS1 (a pBRIDGE derivative expressing the *AP3* protein alone, to asses whether PI is essential for the interaction), or pBRIDGE (to detect false positives). 10 clones were tested: 2 clones (SEP3 and AP1) corroborated recent experiments, showing that they are AP3/PI interactors (Honma et al. 2001), and represent the only identified proteins whose interaction with AP3 is promoted or enhanced by PI (Table 1), 5 clones (PISTILLATA, At1g72740, At1g19100, At1g79150 and At1g04190) were able to interact with AP3 even in absence of PI, 2 clones (At1g22920 and At2g22360) were classified as false positives, since they could trigger the transcription of reporter genes even in absence of AP3 and PI, and 1 clone failed to survive this selection step (At1g28520).

At1g72740 is a member of the MYB transcription factor family. *In vivo* experiments revealed a strong association of this protein with the DNA during every stage of the cell cycle (Koroleva et al, 2005). At1g72740 is the closest homolog in *Arabidopsis* of *Pc*MYB1 from *P. crispum* (Feldbrügge et al., 1997) that is supposed to be involved in the regulation of *P. crispum* Chalcone synthase (*Pc*CHS) gene expression.

At1g19100 is a putative kinase-like ATPase and At1g79150 contains a NOC3 domain, found in proteins involved in the nuclear export of pre-ribosomes; At1g04190 owns a Tetratricopeptide repeat domain (TRP domain). Proteins containing the TRP domain are involved in various functions including cell-cycle, transcription, and protein transport complexes (information retrieved from www.arabidopsis.org annotations).

locus	notes	number of redundant clones	pBRIDGE 5 h	pBRIDGE 8 h	AP3/PI (p3HS3) 5 h	AP3/PI (p3HS3) 8 h	AP3/- (p3HS1) 5 h	AP3/- (p3HS1) 8 h
At5g62350	SEPALLATA3	2	-	-	+	++	+/-	+/-
At1g69120	APETALA1	2	-	-	+	++	-	-
At5g20240	PISTILLATA	13	-	-	+	++	+	++
At1g72740	PcMYB1-like protein	4	-	-	+	++	++	++
At1g19100	putative kinase-like ATPase	1	-	-	+/-	++	+/-	++
At1g79150	NOC3 like protein	1	-	-	+	++	+	++
At1g04190	TRP protein	1	-	-	++	++	++	++
At1g22920	putative JUN Kinase coactivator protein AJH1	3	++	++	+	+	+	+
At2g22360	putative DnaJ protein	2	+	++	+	++	+	++
At1g28520	Unknown protein	1	-	-	-	-	-	-
		30						

Table 1. AP3/PI interactors, isolated in a three-hybrid screening, are listed together with the results of the β-Galactosidase assays. GUS staining was categorized into: very strong (++), strong (+), weak (+/-) and totally absent (-), after 5 and 8 hours of incubation.

3.3.2.1 Expression profile of interactors

The expression profile of *At1g72740*, *At1g04190*, *At1g19100* and *At1g79150* was compared to the one of *AP3* (Figure 19), to test whether their expression domains overlap, hence the interaction is possible *in vivo*. The expression profile of the *Arabidopsis CHS* (*AtCHS*), a homolog of the *PcCHS* that is a putative target of PCMYB1, was also investigated.

The data shown here are based on the data generated in the Weigel department (Max Planck Institute for Developmental Biology, Tübingen): RNA extracted from various organs and tissues at different developmental stages was hybridized to Affymetrix 24K CHIPs (Affymetrix) and signal intensities were determined (Schmid et al., 2004).

At the time this investigation was performed (May 2004), processed data were not available on the Internet. Thus raw data from a selected number of samples (see

Figure 18 for a list) were retrieved from the Internet (www.arabidopsis.org) and processed with RMAexpress, a program that normalizes data and renders them comparable. The output is an excel table, that contains three values for each gene/value coordinate, corresponding to the three replicate hybridizations that have been carried out. The three values were replaced by their median. To facilitate the search of values corresponding to a gene of interest in such a comprehensive excel table, the command “VLOOKUP” was used. Further, these data will be referred to as the “Affymetrix data”.

As one can see from the charts reported in Figure 18 the expression domain of *AP3* overlaps with that of its newly identified interactors, although the latter are not particularly up-regulated in flower.

At1g72740 is weakly and ubiquitously expressed, but *CHS*, its putative target gene, shows a very strong expression in developing flowers and particularly in petals, just like *AP3*. *At1g79150* and *At1g04190* show a surprisingly similar expression profile, partially shared by *At1g19100*, although no significant similarity was found in their amino acid sequence or in their promoters.

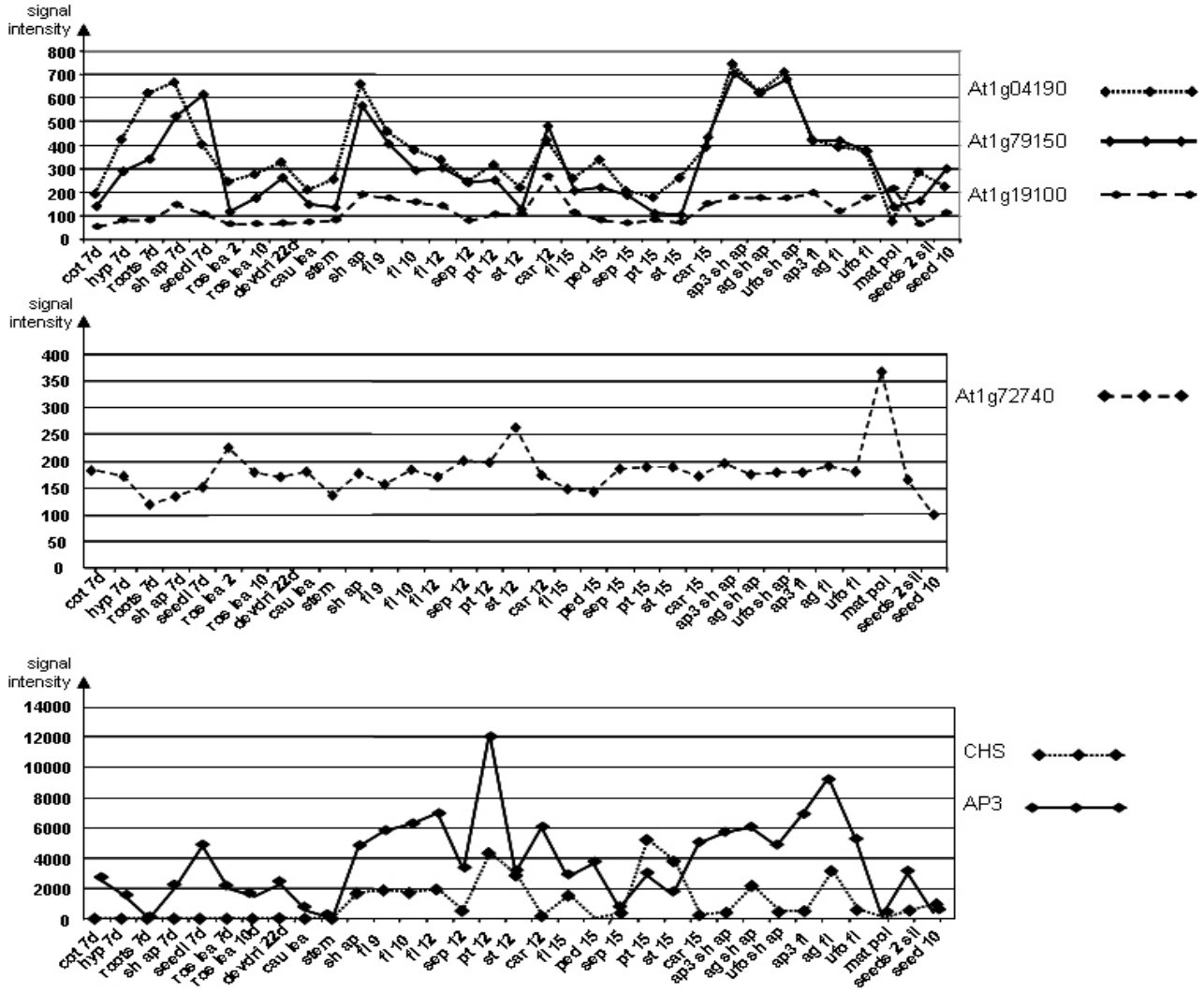


Figure 19. Expression profile analyses of *At1g04190*, *At1g19100*, *At1g72740, At1g79150, CHS* and *AP3,* which are grouped together according to the expression levels. These data have been extrapolated from the Affymetrix data, generated in the Weigel department at the Max Planck Institute for Developmental Biology in Tübingen.

cot 7d	7 days old cotyledons	**fl 9**	stage 9 flowers	**st15**	stamens from stage 15 flowers
hyp 7d	7 days old hypocotyl	**fl 10**	stage 10 and 11 flowers	**car15**	carpels from stage 15 flowers
roots 7d	7 days old roots	**fl12**	stage 12 flowers	**ap3 sh ap**	inflorescence shoot apex from *ap3-6* mutant
s hap 7d	7 days old vegetative shoot apex	**sep 12**	sepals from stage 12 flowers	**ag sh ap**	inflorescence shoot apex from *ag-12* mutant
seedlings	green parts of 7 days old seedlings	**pt12**	petals from stage 12 flowers	**ufo sh ap**	inflorescence shoot apex from *ufo-1* mutant
ros lea 2	17 days old second rosette leaf	**st12**	stamens from stage 12 flowers	**ap3 fl**	flowers from *ap3-6* mutant
ros lea 10	17 days old tenth rosette leaf	**car12**	carpels from stage 12 flowers	**ag fl**	flowers from *ag-12* mutant
devdri 22d	whole rosette leaves 22 days after transition	**fl15**	stage 15 flowers	**ufo fl**	flowers from *ufo-1* mutant
cau lea	cauline leaves	**ped 15**	pedicels from stage 15 flowers	**mat pol**	mature pollen
stem	second internode	**sep 15**	sepals from stage 15 flowers	**seed 2 sil**	stage 2 seeds with siliques
sh ap	inflorescence shoot apex	**pt15**	petals from stage 15 flowers	**seed 10**	stage 10 seeds without siliques.

3.3.3 Ternary complexes-DNA interaction studies

In *Antirrhinum* the MADS box protein SQUA has been shown to increase the DNA binding capacity of DEF/GLO, *in vitro.* Genetic data support this ternary interaction (Egea-Cortines et al. 1999).

In a similar way, in *Arabidopsis* AP3 and PI are thought to form quartets with APETALA1 (AP1), AGAMOUS (AG) and SEPALLATA3 (SEP3) (Theißen and Saedler, 2001), and possibly bind the DNA as tetramers.

In an effort to reproduce protein-protein-DNA interactions in yeast, different constructs have been prepared, expressing class B factors and interaction partners, and their capacity to bind DNA has been tested. To do this, a system, that combines the one-, two- and three-hybrid technologies, was adapted to express up to four proteins (**effectors**) in yeast (yeast strain PJ69-2A), and assay the interaction among the proteins themselves, as well as with DNA fragments (**reporter**) cloned upstream of the HIS3 reporter gene in the vector ycphis3bs (Meijer et al., 1998). If the protein, alone or in combination with the co-expressed ones, is able to bind the DNA upstream of HIS3 and promote its transcription, the yeast cells will grow on media lacking HIS.

200bp and 800bp from the *AP3* promoter and 500bp from the *GLO* promoter were used as **reporter** sequences and cloned upstream of the reporter gene HIS3. These sequences were chosen because *in vitro* studies revealed an interaction between AP3/PI, DEF/GLO and CArG boxes that are present within them (Hill et al., 1998; Tröben et al., 1992).

Versions of DEF, GLO, PI and AP3 without any NLS or GAL4 AD fused to the C-terminus, were produced since a fusion peptide in this position of a MADS box transcription factor could interfere with the protein folding, and hence with the DNA binding activity. Moreover, AP3 and PI are able to access the nucleus as a heterodimer *in planta* (McGonigle et al., 1996), and SEP3 already owns an activation domain known to be functional in yeast (Honma et al., 2001).

As positive control, a previously studied one-hybrid system, where the DNA reporter was cloned in ycphis3bs, was used (supplied by Farnusch Kashani) but not shown.

Apart from the colonies carrying the positive control, no yeast growth was registered in any of the indicated combinations or conditions. Honma et al. (2001) demonstrated

that the constitutive coexpression in *Arabidopsis* of AP3-PI-AP1-SEP3 or AP3-PI-SEP3 or AP3-PI-AP1, was sufficient to activate the expression of a GUS reporter gene cloned downstream of the *AP3* promoter in organs where *AP3* is normally not expressed, like cauline and rosette leaves and roots. Since these scenarios are identical to some of those described here, but the outcome completely different, one could conclude that the novel yeast system, tested here, is not suitable for this type of studies.

effector vectors

name	protein expressed	yeast selection	comments	short reference
p2H	PISTILLATA	URA	a NLS was added to PI N-terminus	**PI**
pGAP3	APETALA3	LEU	GAL4 AD was added to AP3 N-terminus	**AD-AP3**
pGPI	PISTILLATA	LEU	GAL4 AD was added to PI N-terminus	**AD-PI**
pGAP1	APETALA1	LEU	GAL4 AD was added to AP1 N-terminus	**AL-AP1**
p9NT2	SEPALLATA3	ADE	a NLS was added to SEP3 N-terminus	**SEP3**
pAP3E	APETALA3	URA	no NLS was added to AP3	**AP3**
pAP3PI	APETALA3 and PISTILLATA	URA	no NLS was added to AP3 or PI	**AP3/PI**
pAPINLS	APETALA3 and PISTILLATA	URA	a NLS was added to AP3 and PI C-terminus	**AP3/PI-NLS**
pDEF1	DEFICIENS and PISTILLATA	URA	no NLS was added to DEF or PI	**DEF/PI**
pDEGL	DEFICIENS and GLOBOSA	URA	no NLS was added to DEF or GLO	**DEF/GLO**
pSQAD	SQUAMOSA	LEU	GAL4 AD was added to SQUA C-terminus	**SQUA-AD**
pGLAD	GLOBOSA	LEU	GAL4 AD was added to GLO C-terminus	**GLO-AD**
pGSEP3	SEPALLATA3	LEU	GAL4 AD was added to DEF N-terminus	**SEP3-AD**
pDEFAD	DEFICIENS	LEU	GAL4 AD was added to DEF N-terminus	**DEF-AD**

reporter vectors - ycphis3bs derivatives

name	cloned fragment	short reference
p2HAP3	200bp AP3 promoter	**200AP3**
p8HAP3	800bp AP3 promoter	**800AP3**
pGLOp	500bp GLO promoter	**GLOp**
pDHp	500bp DEFH125 promoter	

Table 2. Summary of constructs employed in the novel yeast system described in 3.3.3. Here it is summarised which protein each **effector** construct expresses, and if it is fused to a GAL4 AD or NLS. The **reporter** vectors were tested on appropriate selective media for autoactivation. Only pDHp could activate the transcription of the HIS3 reporter gene *per se,* even at high levels of 3AT, and therefore was excluded from the test.

effectors			reporter
LEU	URA	ADE	TRP
AD-AP3	PI		200AP3
AD-AP3	PI		800AP3
AD-AP3	PI	SEP3	200AP3
AD-AP3	PI	SEP3	800AP3
AD-SEP3	PI	NLS-AP3	200AP3
AD-SEP3	PI	NLS-AP3	800AP3
AD-PI		NLS-AP3	200AP3
AD-PI		NLS-AP3	800AP3
AD-DEF	PI	SEP3	200AP3
AD-DEF	PI	SEP3	800AP3
AD-DEF	PI		200AP3
AD-DEF	PI		800AP3
	AP3/PI	SEP3	200AP3
	AP3/PI	SEP3	800AP3
	AP3/PI-NLS	SEP3	200AP3
	AP3/PI-NLS	SEP3	800AP3
AD-AP1	AP3/PI	SEP3	200AP3
AD-AP1	AP3/PI	SEP3	800AP3
AD-AP1	AP3/PI-NLS	SEP3	200AP3
AD-AP1	AP3/PI-NLS	SEP3	800AP3
AD-AP1	AP3/PI		200AP3
AD-AP1	AP3/PI		800AP3
AD-AP1	AP3/PI-NLS		200AP3
AD-AP1	AP3/PI-NLS		800AP3
GLO-AD	DEF/GLO		GLOp
SQUA-AD	DEF/GLO		GLOp

Table 3. Combination of **effectors** (expressed proteins) and **reporters** (DNA fragments cloned upstream of the HIS3 reporter gene in ycphis3bs vectors; corresponding selective markers are also reported (LEU, URA, ADE and TRP). Every test was conducted in parallel at 22°C and 28°C.

3.4. TARGET PROTEINS of ROXY1 and ROXY2

3.4.1 Isolation of ROXY1 and ROXY2 interactors

The ABCE model explains how homeotic factors interact to promote the development of the right organ in the right position. Class B genes govern the fate of organs in the second and the third whorl, but do not affect where and how many organs are initiated. For instance, the rise of petal primordia is independent of AP3 or PI expression, and other factors, like *RABBIT EARS* (*RBE*) or *PETAL LOSS (PTL)*, seem to be responsible for controlling organ initiation. Recently, *ROXY1* has been shown to be also involved in this process.

A mutation in the *ROXY1* gene causes a reduction in petal number and abnormalities in petal development, indicating a defect in early and late cell division processes (Xing et al., 2005). ROXY1 protein belongs to the glutaredoxin (GRX) family. GRXs are oxidoreductases, reducing disulfide bridges by means of a dithiol or monothiol mechanism. In the monothiol mechanism, GRXs catalyze the reduction of glutathione (GSH) in a process known as deglutathionylation (Rouhier et al. 2004).

GRX-mediated redox reactions are involved in the posttranslational modification of target proteins, by deglutathionylation/glutathionylation or by disulfide bridge reduction. Therefore, the isolation of ROXY1 target proteins is crucial to understand how ROXY1 exerts its function.

Recently, the isolation of Thioredoxin (TRX) or GRX target proteins has been described. Most of the methods focus on *in vitro* approaches: Yamazak et al. (2004) or Motohashi et al. (2001) immobilized the TRX under investigation to a matrix, and used this resin for the thioredoxin-affinity chromatography to isolate proteins that can form disulphide bridges with it; Yano et al. (2001) and Marx et al. (2002) reported a method to identify targets of TRX using subtraction display of the proteins in which cysteines were labelled with a fluorescent dye before or after the incubation with TRX, and then compared on two-dimensional SDS-polyacrylamide gels. In order to detect S-glutathionylated proteins, Fratelli et al. (2002) proposed an *in vivo* approach: cystein labelled with radioactive sulphur (^{35}S) is introduced into the cell under conditions of protein synthesis inhibition; proteins are then extracted from the cells,

separated by gel electrophoresis and detected by autoradiography and sequence analysis. This method was experimented on human cell lines.

Although these methods allow the identification of putative target proteins of TRX or GRX, and test the capacity of the TRX or GRX to form disulphide bridges with their target proteins, or to detect S-glutathionylated proteins *in vivo*, they are time consuming and require the analysis of SDS-gels purified by Matrix-Assisted Laser Desorption Ionization Time-Of-Flight mass spectrometry (MALDI-TOF).

The yeast two-hybrid technology offers a more rapid method to isolate GRXs interactors, and therefore it has been chosen as a first approach to the isolation of ROXY1 interaction partner and target proteins.

ROXY1 and its closest homolog ROXY2, were used as baits in two parallel two-hybrid screenings: *ROXY1* and *ROXY2* coding sequences were cloned into pGBKT7, obtaining pR1 and pR2, respectively.

pR1 and pR2, that responded negatively to an autoactivation test, were introduced into Y187 that was then mated to AH109, carrying an *Arabidopsis* whole plant library cloned into pGADT7-Rec.

The mating products were plated onto selective media, lacking TRP, LEU, ADE, HIS and 1mM 3AT. Out of the about 5000 colonies that were able to grow on the selective media, 200 were picked from each screening and replated on fresh selective media containing 2mM 3AT. A replica of this plate was used to perform a β-Galactosidase assay, to test the expression of the lacZ reporter gene. The library inserts of the survivors of this second selection step that showed GUS staining, were isolated and sequenced (Table 4).

Among the identified ROXY1 and ROXY2 interactors, some were particularly interesting. *TCP3*, *TCP4*, *TCP14* and *TCP21* code for members of the TCP transcription factor family. The name TCP is an acronym derived from the three first characterized proteins of this family: Teosinte Branched 1 (TB1, from *Zea Mays*), CYCLOIDEA (CYC*, Antirrhinum*) and PCF1 (*Oryza sativa*) (Cubas et al., 1999).

Some of the TCP proteins, like CYC, DICHOTOMA (DICH) or CINCINNATA (CIN) from *Antirrhinum*, are involved in the control of cell proliferation. Furthermore, a reduced expression of *TCP3* and *TCP4* in *Arabidopsis* causes deregulation of cell growth in leaves, resulting in a phenotype similar to the *cin* null mutant in *Antirrhinum* (Palatnik et al., 2003; Nath et al*.,* 2003).

SnRK codes for a protein shown to interact with ASK1; the *ask1* mutant phenotype resembles *roxy1,* and ASK1 is supposed to interact with UFO, a positive regulator of AP3 (Farrás et al., 2001; Zhao et al., 1999).

TGA3 is a member of the bZIP transcription factor family and shares high sequence homology with TGA2.2, which interacts in yeast with a putative glutaredoxin encoded by the gene At1g28480 (Mrief Ayed, 2004). Another closely related gene of TGA3, TGA1, has been recently shown to change, in a redox state dependent manner, its DNA-binding affinity (Després et al., 2003).

ROXY1	
At1g06640	2-oxoglutarate-dependent dioxygenase
At1g22070	**TGA3 - bZIP family transcription factor**
At1G23190	phosphoglucomutase
At1g53230	**TCP3**
At1g67090	RuBisCO small subunit 1A
At1G74100	sulfotransferase family protein
At2G04160	AIR3 protein similar to subtilisin-like serine protease
At2G21140	proline-rich protein 2
At3G01090	**SnRK - SNF1-related protein kinase**
At3g12145	polygalacturonase inhibitor
At3g14100	oligouridylate-binding protein
At3g15030	**TCP4**
At3G47620	**TCP14**
At5g08330	**TCP21**
At5G18250	expressed protein
At5G38410	RuBisCO small subunit 3B
AtCg00480	ATPase beta subunit

ROXY2	
At1g03080	kinase interacting family protein
At1g03130	photosystem I reaction center subunit II
At1g10760	starch excess protein (SEX1)
At1g66970	glycerophosphoryl diester phosphodiesterase family protein
At1G79460	ent-kaurene synthetase
At2G04660	E3 ubiquitin ligase
At2g34590	transketolase family protein
At2g47600	magnesium/proton exchanger (MHX1)
At3G01090	**SnRK - SNF1-related protein kinase**
At3G27090	expressed protein
At4G02060	prolifera protein (PRL)
At4G04020	plastid-lipid associated protein PAP
At4g18970	GDSL-motif lipase/hydrolase family protein,
At4g24280	heat shock protein 70
At4g24690	ubiquitin-associated (UBA)/TS-N domain-containing protein
At4g26530	fructose-bisphosphate aldolase
At4G36980	expressed protein
At4G39940	adenylylsulfate kinase 2 (AKN2)
At5g07030	aspartyl protease family protein
At5g08330	**TCP21**
At5g66140	20S proteasome alpha subunit D2
AtCg00480	ATPase beta subunit

Table 4. Interactors of ROXY1 and ROXY2. TCP14 was isolated twice in the ROXY1 screening, and At4g24690 and TCP21 were isolated twice in the ROXY2 screening. The full length coding sequence of proteins highlighted in bold was isolated the interaction with ROXY1 and ROXY2 was re-tested (Figure 20).

Since the DNA clones isolated by the yeast screening were incomplete, full length coding sequences of *TCP3*, *TCP4*, *TCP14, TCP21, SnRK*, *TGA3*, *RBE* and *AmTCP2* were isolated by PCR and cloned into pGADT7. These seven constructs and pGADT7 were transformed into AH109 in combination with pR1, pR2 and pGBKT7. This second interaction test aimed to reproduce the interaction of ROXY1 and ROXY2 with the full length clones. To reveal possible false positives, the empty vector pGBKT7 was used in parallel.

The full length coding sequences of *RABBIT EARS* (*RBE*), a gene responsible for proper petal primordia formation, and *AmTCP2*, a TCP protein from *Antirrhinum*, provided by Farnusch Kashani, were also included in the test. *RBE* was included because the *rbe* mutant phenotype resembles that of *roxy1*, hence it could be involved in the same pathway as ROXY1 and could represent an interactor *in vivo*. *AmTCP2* was included to reveal whether the affinity of *ROXY1* and *ROXY2* for TCP proteins is specific for the isolated TCPs or could be extended to all the TCP members.

The results, reported in Figure 20, show that the interaction of ROXY1 or ROXY2 with the full length version of TCP3, TCP4, TCP21, TGA3.1 and *Am*TCP2 is confirmed, although the latter interacts only very weakly with ROXY2. The interactions with SnRK, TCP14 and RBE cannot be confirmed by yeast two-hybrid because these proteins are able to interact with the GAL4 BD alone.

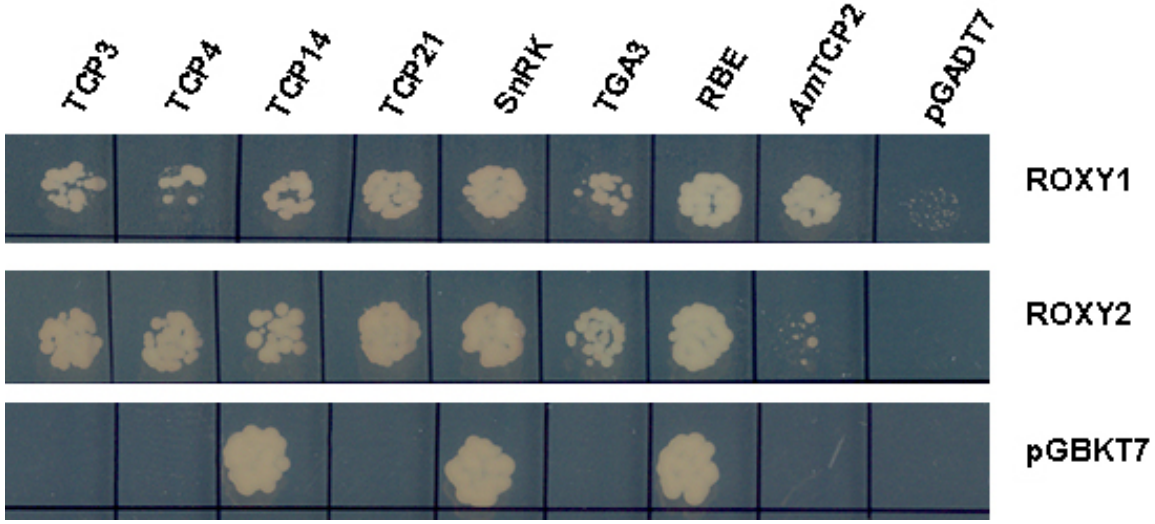


Figure 20. The full length protein of the indicated interactors of ROXY1 and ROXY2, isolated by two-hybrid screenings, and other proteins of interest were tested for interaction with the two baits and the empty pGBKT7 vector.

3.4.2 Expression profile of ROXY1 and ROXY2 interactors

The Affymetrix data allow a rapid investigation of the expression profile of nearly every *Arabidopsis* gene, and were exploited to compare the expression profile of *ROXY1*, *ROXY2* and their interactors (Figure 21).

The expression profiles support the interactions *in vivo*, since their expression domains overlap in “7 days old hypocotyl” and in “shoot apical meristem”, although the expression domains of both *ROXY1* and *ROXY2* is much more specific and restricted to “hypocotyls”, “shoot apical meristems” and “pollen”, and the expression of *TCPs* and *TGA3* is broader.

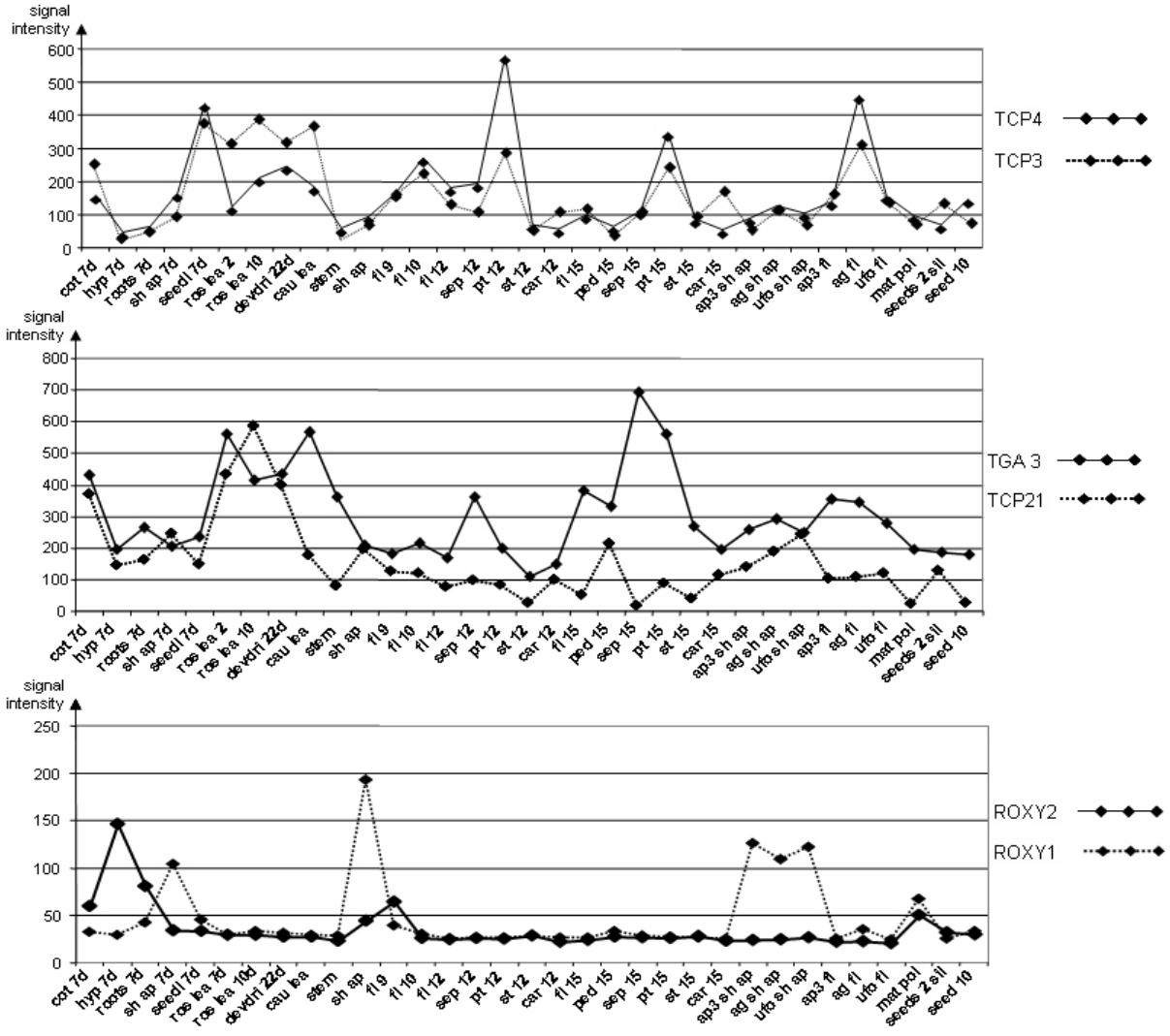


Figure 21. Expression profile of *TCP3*, *TCP4 TCP21*, *TGA3*, *ROXY1* and *ROXY2*, grouped according to expression intensity levels.

3.4.3 Comparison of peptide sequences from TCP3, TCP4, TCP21 and other TCP proteins

The amino acid sequences of TCP3, TCP4 and TCP21 were compared with those of other TCP proteins from *Arabidopsis* and other species.

The TCP domains were aligned with those of CYC, DICH, CIN, TB1, PCF1 and PCF2 (*Oryza sativa*) using a Clustal W online program with the default settings (http://www.ebi.ac.uk/clustalw/index.html). Results are reported in Figure 22, where cysteines, possible targets for the Glutaredoxin mediated redox reaction, are also indicated. For TCP3, TCP4 and TCP21, no other cysteines were found. The cysteines included in the TCP domain are conserved only among members of the clade, suggesting a different regulation mechanism for the TCP proteins. Serines and threonines, potential sites of phosphorylation, included in the TCP domain are indicated in blue (Cubas et al., 1999).

The TCP domain has been shown to have a DNA binding function in the rice TCP proteins PCF1 and PCF2 (Kosugi and Ohashi, 1997), and its secondary structure resembles a basic-Helix-Loop-Helix (bHLH) domain. Nevertheless, TCP and bHLH domains share only the secondary structure, but not the amino acid composition, providing an example of convergent evolution (Heim et al., 2003).

An alignment with all the *Arabidopsis* TCP family members revealed that TCP proteins are grouped in two main clades. The PCF clade, including TCP21, and the TB1/CYC clade, that can be further divided into two groups: the CYC/TB1 group (red) and the CIN group (green), that comprises TCP3 and TCP4 (Cubas, 2004).

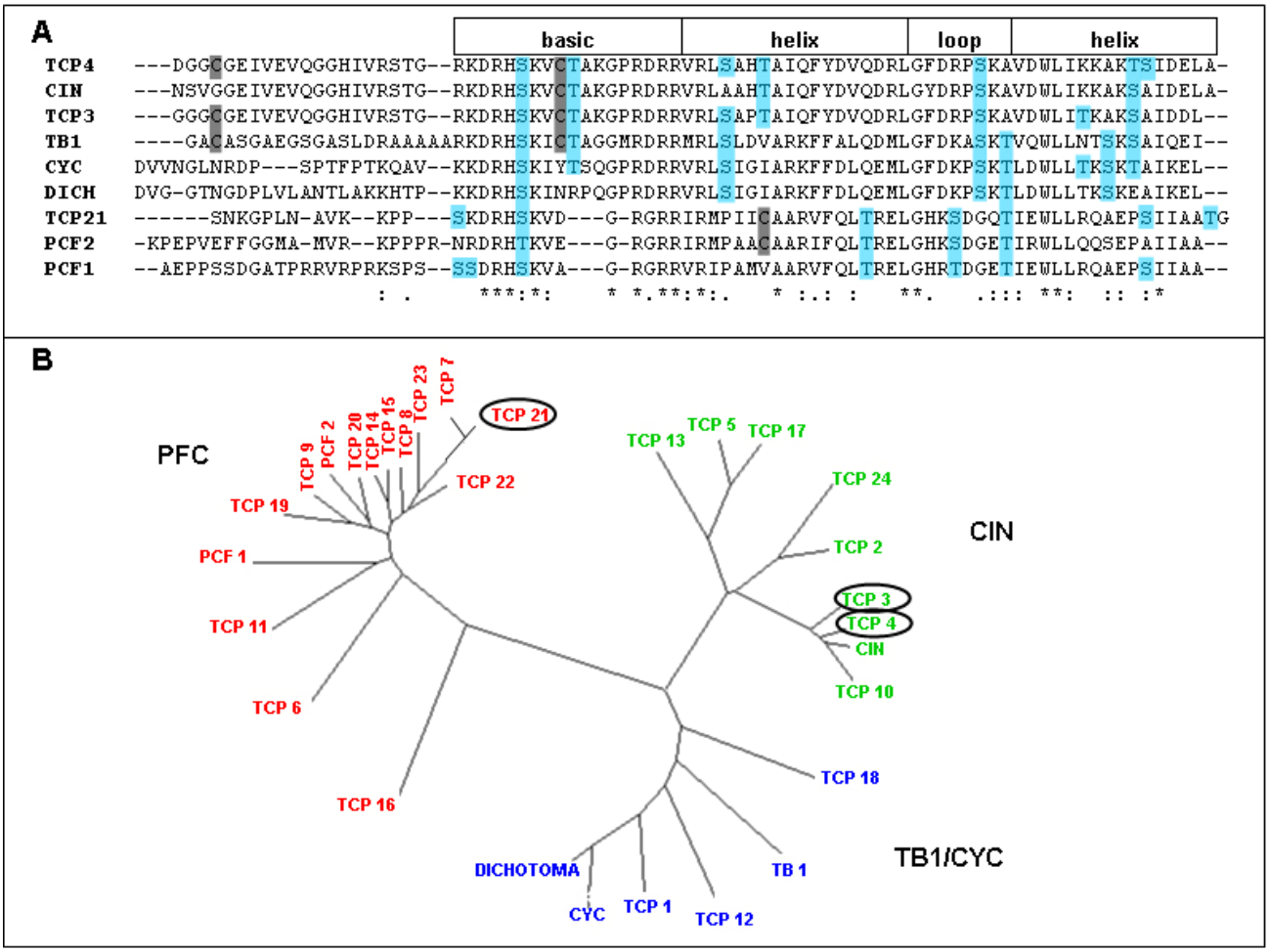


Figure 22. Comparison of the TCP domain from ROXY1 interactors and from other family members.
A) Alignment of the peptide sequence of TCP domains.
B) Tree obtained aligning the TCP domains from all identified *Arabidopsis* TCP proteins and representatives from other species: CINCINNATA (CIN), CYCLOIDEA (CYC) and DICHOTOMA (DICH) from *Antirrhinum*, TB1 (*Zea Mays*) PFC1 and PCF2 (*Oriza sativa*). Three main clades are represented: the PCF clade (red), comprising TCP21, the TB1/CYC clade (blue), and the CIN clade including TCP3 and TCP4 (green). This reconstruction is consistent with a smaller data set, reported by Cubas (2004).

3.4.4 *In situ* expression characterization of the ROXY1 TCP interactors

Although the Affymetrix data supported the TCP proteins-ROXY1 interaction *in vivo* by showing overlapping expression profiles, their resolution is quite low, if one wants to investigate a high complex structure like the flower at early stages of development. So, in order to localize and compare the expression of these genes in a more detailed way, *in situ* hybridizations were carried out with DIG-labelled probes specific for *ROXY1* and *TCP* genes.

This experiment focused especially on young floral meristems (stages 1-3, stages defined according to Smyth et al., 1990), on petal primordia at stage 4 and 5 of developing flowers and, at later stages, on vascular bundles of second whorl organs, because the abnormalities displayed in the *roxy1* mutant are very likely caused by the lack of ROXY1 activity in these areas. Later developmental stages were also investigated.

In situ results are shown separately for *ROXY1*, *TCP3*, *TCP4*, *TCP14* and *TCP21*, from Figure 23 to Figure 27, and are summarized in Table 5.

ROXY1 is expressed in the shoot apical meristem where the flower meristem will rise (Figure 23A). At this stage *TCP3* has a similar expression pattern (Figure 24A), whereas for *TCP4*, *TCP21* and *TCP14* the expression is broader and is detected throughout the meristem (Figures 25A, 26A and 27A). At stage 3, sepal primordia are formed and the expression of *ROXY1* is restricted to a subset of cells that will give rise to petal primordia (Figure 23A). At this stage, the transcript of the investigated *TCP* genes is detected in all the cells forming the floral meristem (Figures 25A-27A).

At stage 5 organ primordia became distinguishable. The expression of *ROXY1* from this stage to stage 8 is restricted to petal and stamen primordia, particularly restricted to vascular bundles (Figures 19A and 19B). At these stages, the expression of *TCP3* and *TCP14* was detected in petals, stamens and carpels (Figures 24B, 26B and 26C), whereas *TCP4* and *TCP21* expression was broader, extending into sepals (Figures 25B, 25C and 27B).

From stage 9 on, the *ROXY1* signal becomes restricted to the central vasculature of petals and ovules (Figures 23C and 23D). During the late developmental stages, the expression of *TCP* genes becomes more differentiated: *TCP3* is detected on the petal tip only (Figure 28C), *TCP4* is detected in sepals, petals and ovules (Figures 25D and 25E), and *TCP21* remains ubiquitously expressed in the four floral organs and ovules (Figures 27C and 27D). *TCP14* mRNA was detected in ovules and in abaxial sepals (Figures 26D and 26E). The detection of *TCP14* in abaxial sepals and not in adaxial sepals is noteworthy. Sepals are organs that show a slightly asymmetric growth, being the abaxial ones longer and overgrowing the adaxial ones. The asymmetric expression of *TCP14* might be correlated to asymmetric organ development, as shown for *CYC* and *DICH*.

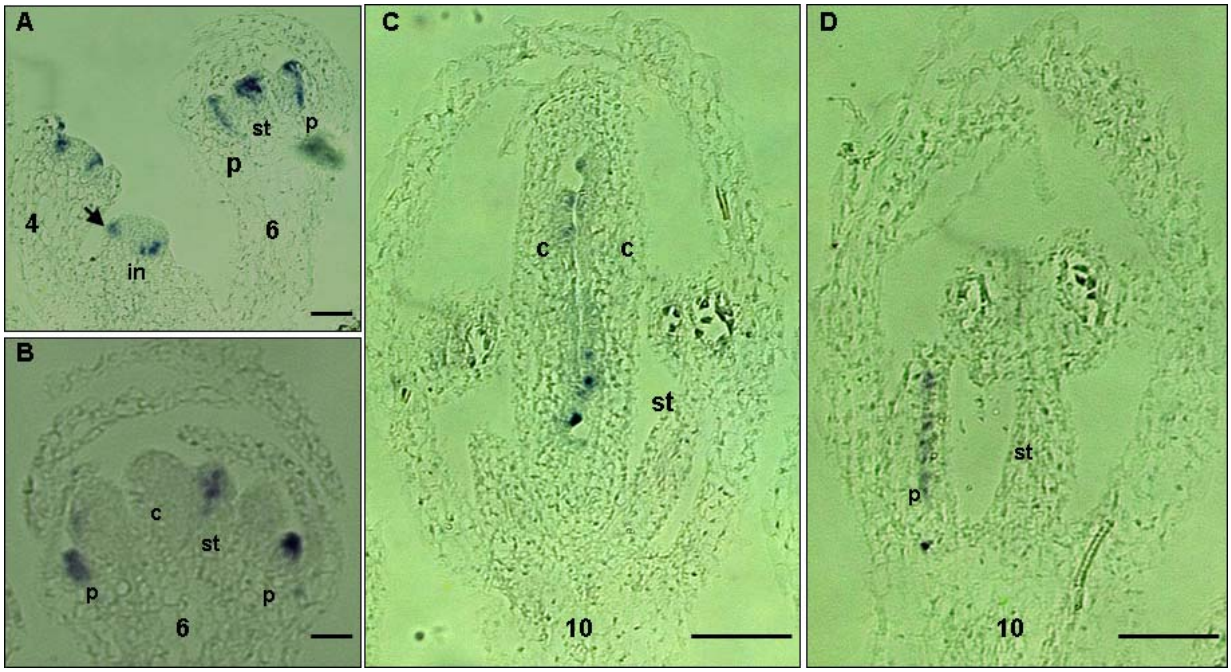


Figure 23. *ROXY1 in situ* hybridization.
A) *ROXY1* is expressed in the shoot apical meristem where the flower meristem will arise (arrowhead), and in the flower meristem at early stages where petals will arise (flower stage 4). It is very likely that *ROXY1* contributes to the development of the right number of organs in this domain. *ROXY1* is also expressed in developing petals and stamens at later stages, mainly restricted to vascular bundles (flower stage 6).
B) Flower at stage 6 showing *ROXY1* expression in petal and stamen primordia.
C) and D) At later stages *ROXY1* is expressed in vascular bundles of petals and in ovules.
Bar = 50 µm in A-B and 100 µm in C-D

s, sepals; **p**, petals; **st**, stamens; **c**, carpel; **cl**, cauline leaves; **in**, inflorescence apex; **ad**, adaxial side; **ab**, abaxial side. Numbers indicate developmental stages defined according to Smyth et al. (1990).

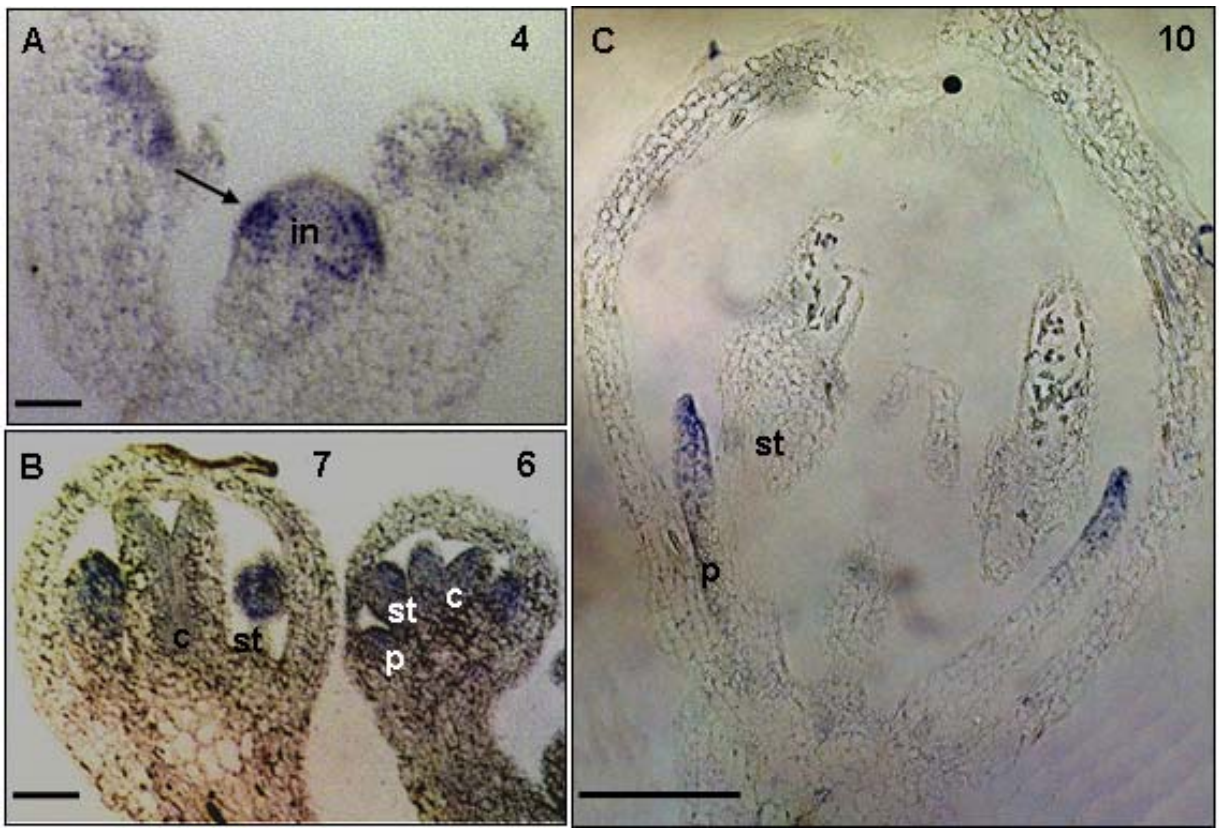


Figure 24. *TCP3 in situ* hybridization.
A) *TCP3* is expressed in the inflorescence apex where a new primordium will rise. At an early stage of flower development it is expressed throughout the floral primordia, and particularly in the outermost whorls.
B) *TCP3* is expressed in petals, stamen and gynoecium primordia. Its expression is maintained as the flower develops.
C) At late stages the expression of *TCP3* is restricted to the tip of petals.

Bar = 50 μm in A-B and 100 μm in C

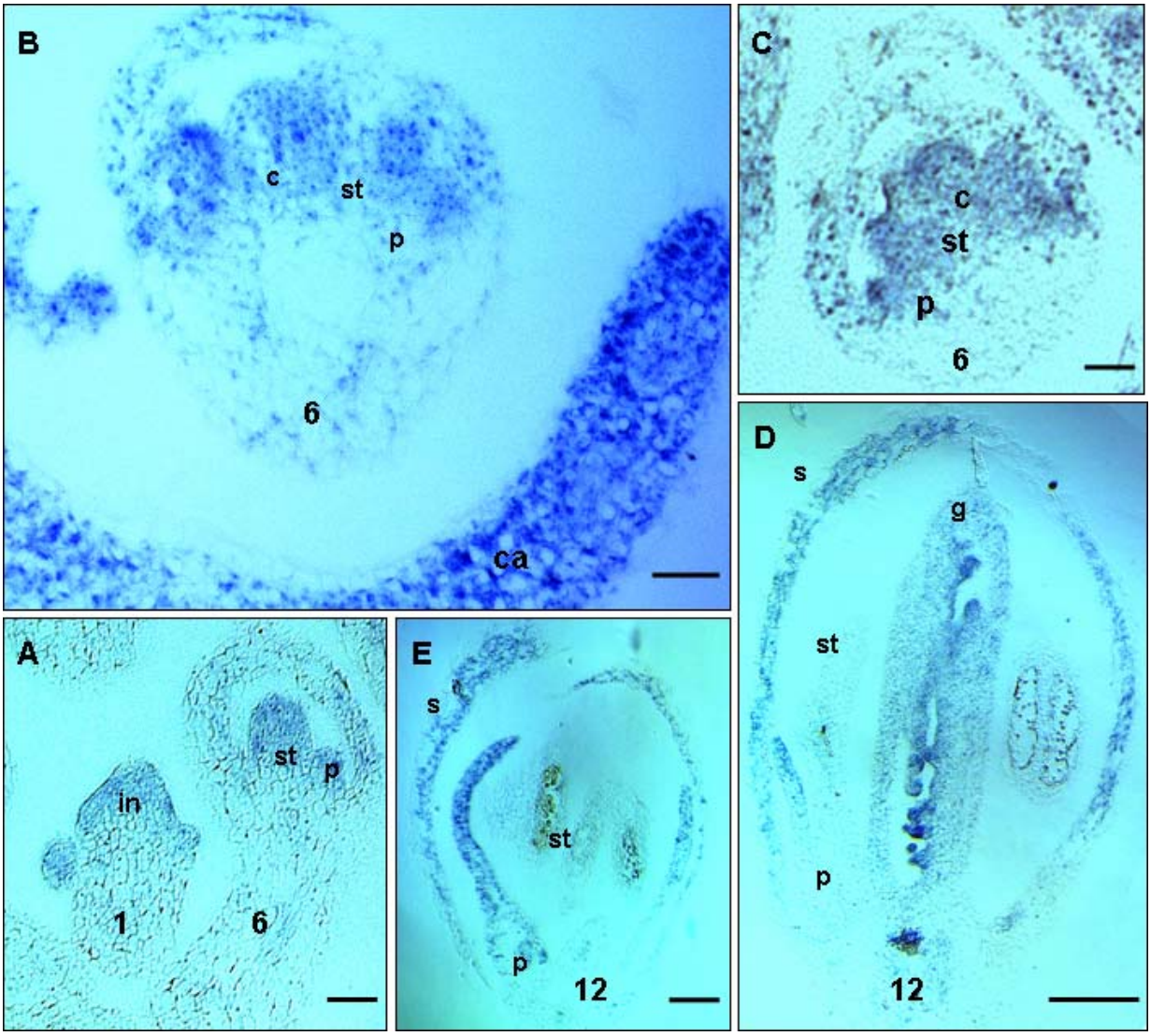


Figure 25. *TCP4 in situ* hybridization.
A) *TCP4* transcripts are detected throughout the inflorescence apex and young flower meristems.
B) At later stages of flower development, transcript is visible in the four organ primordia and in cauline leaves (C). It seems that *TCP4* transcript accumulates only in the nucleus in flowers cells, whereas in cauline leaves it is detectable throughout the cell.
D and E) In older flowers *TCP4* is maintained at high levels only in sepals, petal tips and ovules.

Bar = 50 µm in A-C and 100 µm in D-E

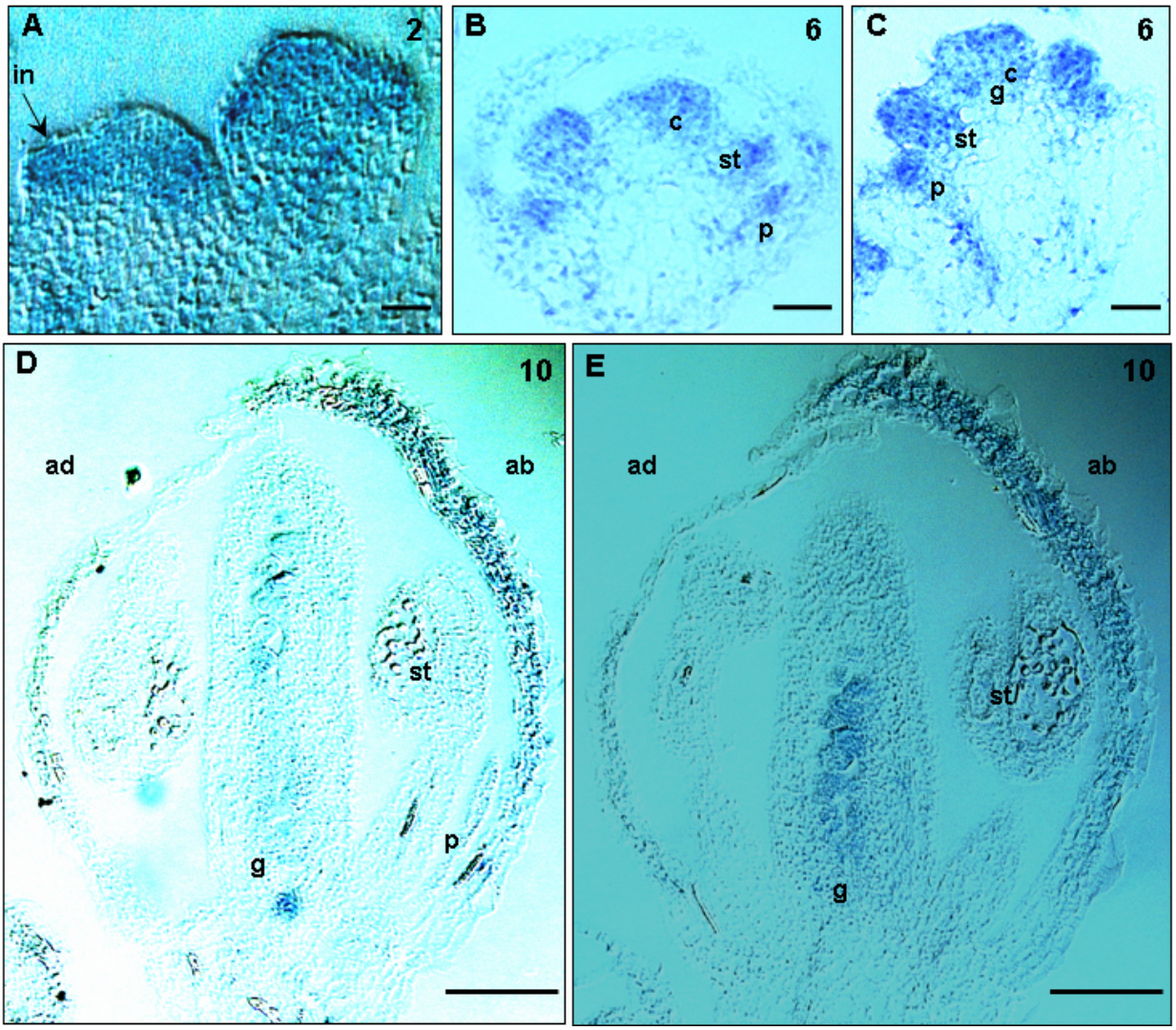


Figure 26. *TCP14 in situ* hybridization
A) *TCP14* is expressed in inflorescence meristems where a new flower will rise (arrowhead); and is detectable throughout the flower meristem.
B) and C) *TCP14* is expressed in the four organ primordia.
D) At later stages, transcript accumulates in ovules and in sepals, and particularly in the tip of the abaxial one, but was not detectable in petals.

Bar = 50 µm in A-C and 100 µm in D-E

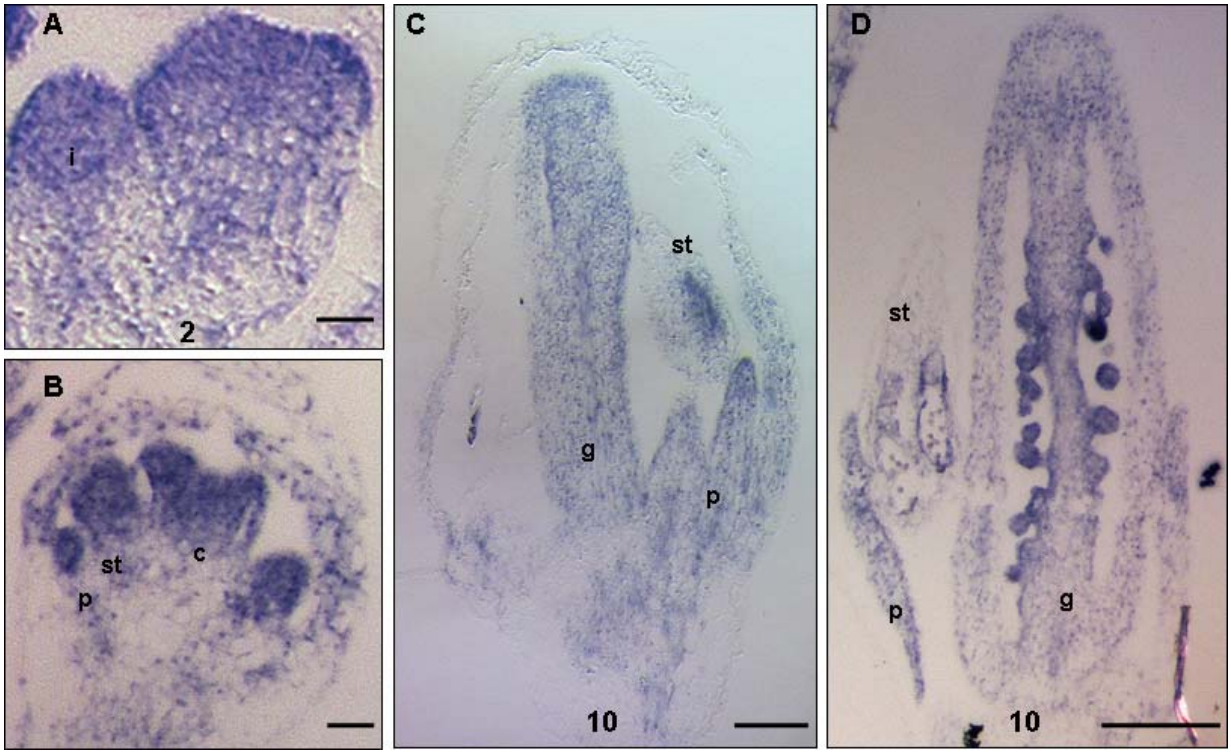


Figure 27. *TCP21 in situ* hybridization.
A) and B) The expression of *TCP21* appears almost ubiquitous. Onset of expression is detected at early stages in flower development; expression is maintained throughout flower development in the four organs and ovules (C and D).

Bar = 50 µm in A-B and 100 µm in C-D

		ROXY1	***TCP3***	***TCP4***	***TCP14***	***TCP21***
	SAM	++	++	+	+	++
	flower meristem	++	++	+	+	++
young buds	Sepal	-	-	+	-	++
	Petal	++	+	+	++	++
	Stamen	++	+	+	++	++
	Carpel	-	+	+	++	++
old buds	Sepal	-	-	++	+	+
	Petal	+	++	+	-	++
	Stamen	+	-	-	-	++
	Carpel	-	-	-	-	++
	Ovule	+	-	++	+	++

Table 5. The expression domain of *TCP* genes and *ROXY1* is summarized and compared. (-) no expression was detected; (+) weak expression (++) strong expression.
SAM: Shoot apical meristem; **flower meristem**: buds until stage 4; **young buds**: flowers from stage 5 to 8; **old buds**: flowers from stage 9.

CONCLUSIONS

The defect in primordia formation and development registered in *roxy1* is due to lack of ROXY1 activity in floral meristems and petal primordia.

The *in situ* analysis of *ROXY1* interactors, the *TCP3*, *TCP4* and *TCP21* genes, revealed an overlapping expression domain in the inflorescence meristem and in the flower at early developmental stages (Table 5). The expression of *TCP3* in particular is very similar to that of *ROXY1* in the inflorescence and floral meristems.

These expression data support that TCP3, TCP4 and TCP21 might interact with ROXY1 *in vivo*.

4. DISCUSSION

Two different groups of factors are involved in the process of floral organ formation: the first one induces the formation of organ primordia with the right positioning and timing, and the second one determines its identity during further development.

Genes like *RBE*, *PTL* and *ROXY1* belong to the first group: their contribution is essential for the development of the right number of petals in the second whorl, but if they are mutated, organ identity is not affected.

Members of the second group are also known as homeotic genes; they interact according to the ABCE model in the control of flower organogenesis.

Evidence from different organisms shows that the homeotic genes, most of them encoding MADS box transcription factors, interact physically to form tetramers that are able to bind DNA.

In the work reported here, the X-ChIP technique has been employed to investigate interactions of class B transcription factors with putative target sequences, and the yeast system has been used in an attempt to reproduce the quartet-DNA complexes. Furthermore, an effort was conducted to study the formation of MADS box tetramers and their ability to bind the DNA *in vivo* and in yeast.

Finally, to study the floral organ primordia initiation process, putative interactors of ROXY1, that might participate in setting the proper number of second whorl organs, were identified.

4.1 *In vivo* studies: the X-ChIP technique

4.1.1 X-Chip: preliminary considerations

The X-ChIP technology aims to demonstrate that a postulated DNA-protein interaction occurs *in vivo*. This technique can be divided into two steps: the first one concerns the fixation of proteins to the DNA and the precipitation of the DNA-protein complexes with a specific antibody. In the second step, the immunoprecipitated DNA, that is enriched for specifically bound target sequences, is analyzed by SQ-PCR.
The first step was carried out following roughly an established protocol (Müller, 2004; Wang et al., 2002; Wang et al., 2004).
For the second step, the procedure reported in the literature (Orlando et al., 1997; Orlando, 2000; Wang et al., 2002; Köhler et al*.,* 2003; Wang et al., 2004; Dilusha et al*.,* 2004) was improved by including different control levels, as discussed below.
These controls allowed to discriminate between real positive and false positive interactions, which will be demonstrate by the comparison of two X-ChIP experiments. Both experiments aim to demonstrate that the *AP3* promoter is bound by DEF in the *ap3-1*TDEF system. They were carried out in two different ways: the first (reported in Figure 28 and Figure 17) was performed with the new procedure and the second (reported in Figure 29) according to the literature standards. The output is different: in the first case the interaction cannot be confirmed, in the second case the interaction seems to be confirmed. Below, an explanation for this discrepancy is given.
By applying the new procedure for the X-ChIP data interpretation, it was calculated that the enrichment of the *AP3* promoter (area 6 in Figure 28 and Figure 17) vs. background control (area 7 in Figure 28 and Figure 17) was the same either when *ap3-1*TDEF or *ap3-1* material was used; hence it was concluded that the DEF-*AP3* promoter *in vivo* interaction is not demonstrable.
Figure 28 shows a detailed report of each replicate of the experiment. The values reported correspond to band intensities after being normalized according to the primer strength (step 3, 3.1.2.1) and to the total amount of DNA in the IPed pool (step 4, 3.1.2.1). Average and standard deviation are also reported.

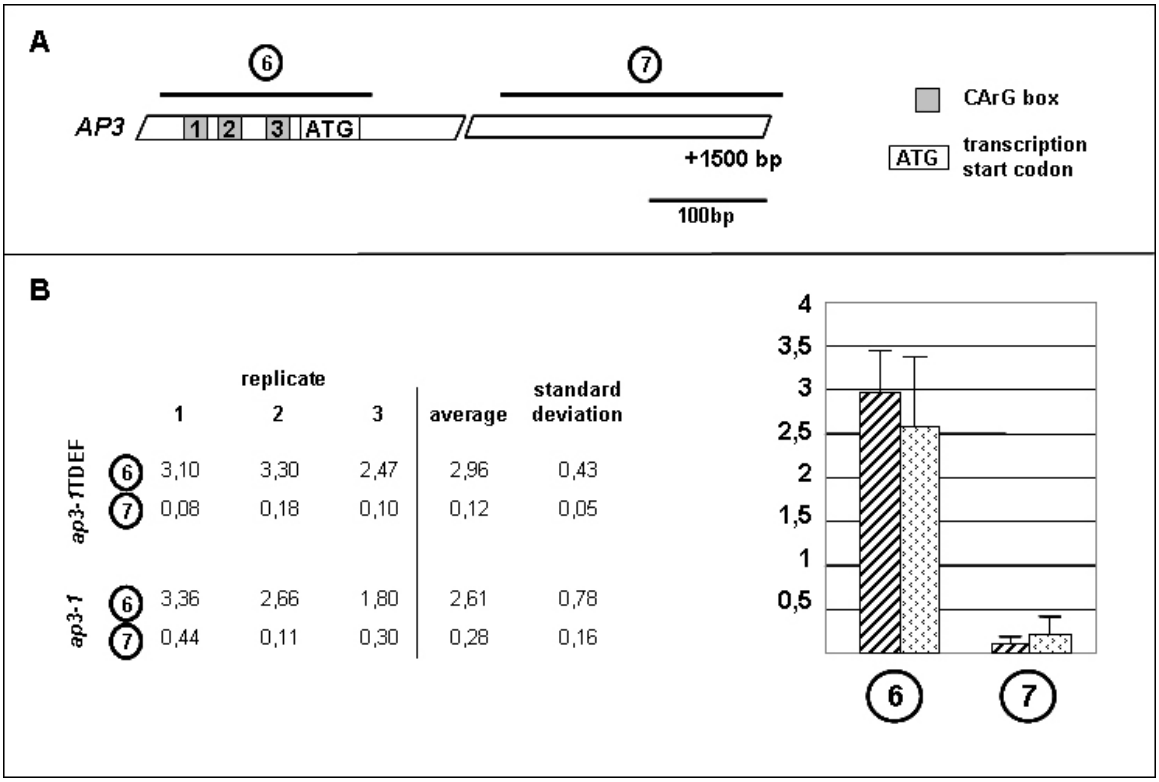


Figure 28. X-ChIP experiments on *ap3-1*TDEF to investigate the *AP3* genomic locus.
A) Investigated areas on the *AP3* genomic locus: region 6 is close to the ATG start codon and includes CArG boxes that are bound by AP3/PI *in vitro* (corresponds to region 6 in Figure 17); region 7, that in this experiment represents the background control, is in the sixth intron in the *AP3* genomic locus and is located about 1500bp downstream of the ATG start codon (corresponds to region 7 in Figure 17).
B) Relative enrichment of the areas 6 and 7 from three independent replicates of the experiment. These values correspond to SQ-PCR band intensities after normalization steps. The average and standard deviation were calculated and reported in the chart on the right hand side.

Figure 29 shows the experiment performed according to the literature standards. In this experiment the background control is represented by *TUB2*, a sequence that should not be immunoprecipitated (Wang et al*.,* 2002). The relative enrichment of the *AP3* promoter vs. the background control was measured for four samples:

wt-se: wild type-like material precipitated with a specific serum.

wt-pre: wild type-like material precipitated with a pre-immune serum.

m-se: mutant material precipitated with a specific serum.

m-pre: mutant material precipitated with a pre-immune serum.

In the case reported here the wild type-like was represented by *ap3-1*TDEF, the mutant by *ap3-1* flowers from plants grown at 26°C and an anti-DEF serum was employed.

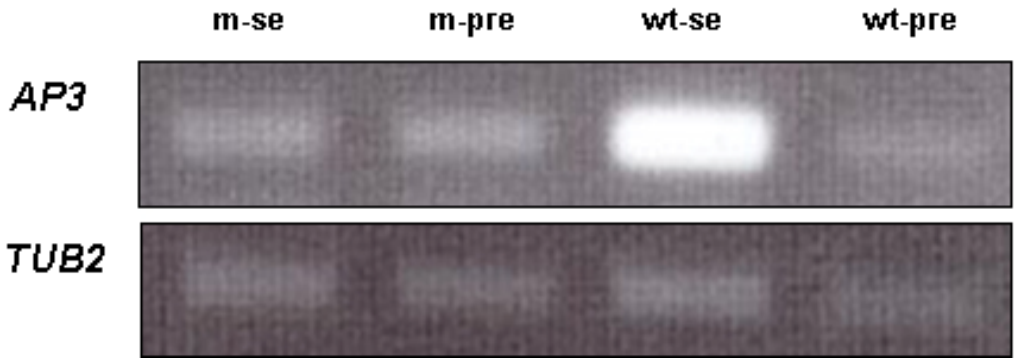


Figure 29. Specific primers for the *AP3* promoter and *TUB2* were used to perform PCRs on DNA immunoprecipitated with anti-DEF serum and pre-immune serum, from *ap3-1*TDEF and *ap3-1* plants.

Considering the results reported in Figure 29, one could conclude that the *AP3* promoter is directly bound by DEF, because the *AP3* band seems to be enriched in wt-se with respect to any other sample and to the background control *TUB2*. *TUB2*, used to normalize samples by Wang et al. (2002), showed the same enrichment.

The procedure followed in the experiment reported in Figure 28 and Figure 17, and the experiment output obtained by applying it, was considered to be more reliable for at least four reasons:

1) Trying to quantify DNA bands only considering the gel pictures, especially weak bands like those reported in Figure 29 for *TUB2* and *AP3* m-se, m-pre and wt-Pre, can result in a rough and not precise estimate of values, and slight differences can easily be overlooked. DNA products should be quantified in an objective way, preferentially by an instrument such as a PhosphorImager scanner.

2) A gel picture always shows only one experiment replicate. The reliability of X-ChIP experiments is based on reproducibility. Replicates of the same experiment should be comparable and a statistical analysis of data should be carried out. These steps are not possible considering gel pictures only.

3) The region bound by the protein under investigation should be enriched with respect to a background control, represented by *TUB2* in Figure 29 and region 7 in Figure 28 and Figure 17. It would be advisable that the background control were located within a few thousand base pairs from the binding site, where the chromatin density is the same. In fact, due to different chromatin density, various genomic areas are not equally accessible to the PFA and antibodies, and thus could respond

differently to the fixation and immunoprecipitation. For these reasons region 7 is considered a more reliable background control than *TUB2*.

4) In order to compare band intensities obtained with different primers, such as "*AP3*" and "*TUB2*", one should consider that the characteristics of the primers themselves and of the amplified sequences influence the PCR yield. That is why a normalization step, employing the primers on a non immunoprecipitated sample (e.g. "Input DNA"), is necessary.

Considering these four points one can understand why results reported in Figure 29 are misleading: the *AP3* band in wt-se is stronger than any other band because the total amount of DNA in the wt-se sample is probably higher than in the other samples, as registered in many other experiments (Figure 7). This consideration was corroborated by SQ-PCRs conducted on adapter ligated IPed DNA with primers annealing on the adapter: the DNA amplified from wt-se was definitely more than in the other samples (not shown). The *TUB2* band corresponding to the wt-se sample, taken as an indicator of its total amount of DNA, does not reveal this difference for two reasons: first, because the *TUB2* band is not quantified, thus an unbiased normalization cannot be performed; second, because one single experiment replicate can be misleading, and, as discussed, results should be confirmed by statistics.

A more thorough investigation, conducted with the procedure applied in the example shown in Figure 28, could reveal that the ratio *AP3* promoter/*TUB2* background control is similar in wt-se and in m-se, as shown for *AP3* promoter/region 7 background control (Figure 28).

The way of investigation shown in Figure 28, although more reliable than that reported in Figure 29, could still be improved: the putative binding region was compared only with one other region contained in the genomic locus of *AP3*. The comparison with more regions of the surrounding genomic locus circumvents the high variability of PCR amplification and renders the system more reliable. Furthermore, the construction of a bell shaped curve allows the identification of a climax, corresponding to the binding site.

These considerations have been taken into account for the investigation of the *NAP* promoter in *Arabidopsis,* and for the *DEFH125*, *EXTENSIN* and *GLOBOSA* promoters in *Antirrhinum*, where at least four promoter regions were investigated.

4.1.2 SQ-PCR reproducibility

The X-ChIP technique relies on SQ-PCR to display an enrichment of genomic sequences. In a SQ-PCR, conditions should be optimal: the cycling should be stopped when the reaction is still in the exponential phase and results should be reproducible. Preliminary tests were carried out to asses within which range a PCR can still be considered to be in the exponential phase. All subsequent PCRs were performed according to the standard set in these tests.
To limit the variability of the PCR and give reliable primer strength normalization, PCRs, performed using “input DNA” as template, were conducted at least five times (not shown). The calculated median value was used in the normalization step.

4.1.3 Isolation of new target genes by the X-ChIP technique

The possibility to isolate unknown DNA sequences, representing regulatory genomic DNA fragments from target genes by the X-ChIP technique, has been described in the literature. Towards this goal, the authors followed basically two approaches: Chromatin double immunoprecipitation (Nelson et al., 2004) and ChIP-to-CHIP (Weinmann et al., 2002; Bing Ren et al., 2000; Iyer et al., 2001; Martone et al., 2003). The first approach was also applied in this work with the intention to isolate new DEF/GLO target genes. The second one demands the use of a genomic DNA array, which, considering the rapid progress in the plant genomic field, might become accessible in the near future.

4.1.3.1 Chromatin double immunoprecipitation

In an effort to isolate new targets of DEF/GLO, a tandem double immunoprecipitation was carried out on chromatin extracted from stage 3 petals. Anti-DEF and anti-GLO sera were used successively, in order to reduce the amount of unspecifically immunoprecipitated DNA and to increase the chance of isolating new target sequences.
Seven DNA fragments were isolated and sequenced. Clone 14, that contained a putative CArG box (CCATAATTGA), was enriched with respect to the background in

an independent X-ChIP experiment with specific primers when petal material was used. No enrichment was reported with material from "*def-gli* mutant flowers". In this experiment, conducted in a single replicate, the *DEF*-CArG region was included as a positive control because it was carried out before a complete analysis of the *DEF* genomic locus, including six replicates, was completed. The *DEF*-CArG vs. *DEF*-STOP enrichment was registered in the wild type sample only, and not in the mutant. This observation contradicts what has been reported in the results chapter (3.2.1.2 and Figure 9) and thus reveals the necessity to conduct replicate experiments.

The interaction of DEF with the 1500bp upstream and 1500bp downstream of the clone 14 has not been investigated yet; thus, no bell shaped curve was drawn. The identification of a bell shaped curve would allow to locate the DEF/GLO binding site, corresponding to the climax, that might coincide with the CArG box contained in the clone 14. Sequencing 3000bp of adjacent regions did not reveal any gene or coding region and did not significantly match any known genomic sequence, neither could it be classified as short tandem repeat (STR) or microsatellites. The genes that DEF/GLO potentially regulates through the interaction with clone 14 could be further away than 1500bp: although most of the regulatory elements are included in the gene promoter and close to the start codon, some genes have been described to be regulated by more distant elements. For instance, *AGAMOUS* expression is regulated by an element located over 2000bp downstream of the ATG start codon and that is part of the 3000bp long second intron (Deyholos et al., 2000).

In order to elucidate this question, further sequencing of the area surrounding clone 14 is required. The transcription of the closest coding regions will be characterized in the wild type and in the inducible *def101* mutant, to assess if it is regulated by DEF/GLO.

The finding that one region out of seven isolated is likely bound by DEF/GLO was surprising. In fact, calculating the chances to isolate one, the matter could be simplified considering the genome of *Antirrhinum* (ca. 430 Mb) as divided in one million fragments, each about 400-500 bp long. In the IPed pool the whole genome is represented, but the specifically immunoprecipitated fragments are theoretically 28 times as concentrated as any other species (assuming that the *DEF*-CArG/*DEF*-STOP represents the "specifically immunoprecipitated"/"back-ground" ratio).

If so, the chance (P) to isolate a specifically immunoprecipitated fragment would be "P=28*nsf/1.000.000", where nsf is the number of specifically immunoprecipitated species, i.e. DNA fragments bound by the protein under investigation. Bey et al. (2004) predicted that about 200 genes are regulated by DEF/GLO in petals at stage 3. Assuming that all of them are directly regulated through a DEF/GLO-DNA interaction, nsf would be 200 and P would correspond roughly to one in two hundred (P=0,005).

In the experiment reported here, one sequence out of seven was actually a target of DEF/GLO. Thus a rough estimation of the probability would be 1/7=0,14.

If this is true the nsf is about 5000, a number much higher than what Bey et al. (2004) or other authors (Zik et al., 2003; Wellmer et al., 2004) indicated as possible class B factors targets. However, these data should be considered carefully because the probability discussed above and the 28 times enrichment after the double immunoprecipitation, are results from one single experiment.

One explanation for this discrepancy is that the DEF/GLO-DNA interaction could not always be functionally relevant and affect target gene transcription: the heterodimer could interact with any suitable CArG box if the chromatin conformation allows it. Martone et al. (2003) showed that about 38% of the genes they reported to be bound by p65, a human transcription factor, do not have their transcriptional activity altered by the presence of p65. This could be the case of the DEF/GLO-clone 14 interaction, yet not related to any gene expression.

Combining these data, the chance to isolate a functional protein-DNA interaction by X-ChIP might be very low. Therefore, the canonical use of this technique, i.e. to demonstrate a putative direct regulation of a target gene, is more convenient.

4.1.3.2 ChIP-to-Chip technique

The ChIP-to-Chip technique has been recently described as a procedure that combines X-ChIP technology with genomic DNA arrays to isolate new target genes (Weinmann et al., 2002; Bing Ren et al., 2000; Iyer et al., 2001; Martone et al., 2003). In this technique, the immunoprecipitated DNA is hybridized to array filters onto which genomic DNA regions were spotted.

The comparison of hybridization patterns, carried out with DNA immunoprecipitated from mutant and from wild type tissues, would allow the identification of differentially hybridized spots, corresponding to target sequences.
This technology allows a rapid identification of target genes, although one should consider that genomic microarrays are currently not commercially available for many organism, such as *Arabidopsis* and *Antirrhinum*. Furthermore, as discussed above, the identification of a binding site should be followed by a transcriptional characterization of the putative target gene in the wild type and in the mutant, as the functional link between the protein/DNA interaction and its biological relevance is not immediately clear.

4.2 Results of X-ChIP experiments

Two genes of *Arabidopsis* and four of *Antirrhinum* were analyzed by X-ChIP as possible targets of AP3/PI and DEF/GLO, with the new procedure for data analysis. For *DEFH125* the interaction with DEF was proved by X-ChIP. A DNA-protein interaction was not demonstrable for the other genes. The results for the different investigated genes are discussed below.

4.2.1 The DEF/GLO-*GLO* and *DEF* promoter interaction cannot be proved by X-ChIP

The results reported cannot confirm the interaction of DEF/GLO and the promoter of *GLO* and *DEF*. Although preliminary X-ChIP experiments supported this hypothesis (Müller, 2004), a careful analysis of the data revealed that for *GLO* the same “binding site vs. background” enrichment was reproduced in absence of DEF, and a similar bell shaped curve could be extrapolated from both wild type and mutant data.
The flanking regions of the area containing the CArG box in the *DEF* promoter were not investigated by X-ChIP experiments. In fact, the DNA sequence upstream of the CArG box is not known and two inverted repeats located downstream of the CArG box inhibit the PCR. Hence no bell shaped curve was extrapolated for this genomic locus, and the DEF/GLO-*DEF* promoter interaction could not be proved by X-ChIP.

What could have misled previous investigations (Müller, 2004) is the fact that SQ-PCR products from wt-se, wt-pre and m-se, were compared directly, without a normalization step.

On the other hand, these data do not demonstrate that the interaction of DEF/GLO and *GLO* or *DEF* promoter does not occur *in vivo*, but they show that the X-ChIP technique, at this condition, is not suitable for this purpose.

To improve the X-ChIP protocol and make it suitable for the detection of DEF/GLO-*GLO* promoter interaction, three attempts were conducted (data not shown):

1) Using an anti-GLO serum (Zachgo et al*.,* 1995), instead of the anti-DEF one.

2) Making conditions more stringent by performing two tandem immunoprecipitation steps: the first with an anti-DEF serum and the second with an anti-GLO one.

3) Employing a purified anti-DEF serum that was run through columns, where extracts from plant tissues not expressing DEF, were fixed to a solid matrix. In this step, unspecific antibodies in the serum, that could contribute to increase the background, were reduced.

No significantly different results were obtained when these parameters were changed.

An explanation for the failure to demonstrate a DEF/GLO-*GLO* promoter interaction is the cross-reactivity of the employed sera with other factors present on the *GLO* promoter, even when the *GLO* gene is not actively transcribed, like in the *def-gli* mutant. One should consider that the DNA immunoprecipitated from the mutant is less than from wild type material; the proportional reduction of the background and the lack of high affinity DEF epitope-antibody interactions could facilitate the formation and detection of weak protein-antibody interactions, like the unspecific one described here.

In order to investigate whether this weak interaction is organ or whorl specific, a single X-ChIP experiment replicate (not shown) was conducted on different parts of the mutant flower (first whorl sepals, second whorl sepals and carpels) with the anti-DEF serum. The bell shaped curve was obtained only for second whorl sepals, suggesting that the anti-DEF crossreacts with proteins expressed in the second whorl. Likely, these could be other MADS box proteins, such as SQUA, that is thought to participate in the interaction of DEF/GLO with target sequences (Egea-Cortines et al*.,* 1995), or DEFH24, DEFH52, DEFH72 and DEFH200, that have been reported to

be expressed in petals (Davies and Schwarz-Sommer, 1994; Schwarz-Sommer et al., 2003; Davies et al., 1996).

The unspecificity of the used serum could be one of the causes that renders the X-ChIP technique unsuitable for the detection of many protein-DNA interactions, as it is discussed for the *ap3-1*TDEF system (see below).

4.2.2 The DEF/GLO-*EXTENSIN* promoter interaction cannot be proved by X-ChIP

EXTENSIN was shown to be upregulated by DEF/GLO (Bey et al., 2004), and preliminary X-ChIP data (Müller, 2004) suggested that the heterodimer interacts directly with the *EXTENSIN* promoter. Data presented here do not confirm a direct *in vivo* interaction, because no enrichment was registered for any investigated area belonging to 5kbp of an isolated EXTENSIN genomic locus, neither in the wild type, nor in the mutant. However, X-ChIP data do not exclude that the interaction between DEF/GLO and the *EXTENSIN* promoter could occur in the investigated area, *in vivo*.

One should consider that the expression domain of *EXTENSIN* in petals is restricted to epidermal cell layers (Bey et al., 2004), that constitute about 25% of the organ, as *Antirrhinum* petals are composed of 8 cell layers. Assuming that the DEF/GLO-*EXTENSIN* promoter interaction occurs *in vivo* only in cells where *EXTENSIN* is expressed, the specifically immunoprecipitated DNA fragments from *EXTENSIN* would be 1/4 of the specifically immunoprecipitated DNA fragments from another target gene, bound throughout the organ. Therefore, the PCR product corresponding to the real binding site, contained in the *EXTENSIN* promoter, might not be distinguishable from the background.

This consideration, which will be referred to as the dilution of specifically immunoprecipitated DNA fragments, would explain why the X-ChIP technique might not be suitable to demonstrate interactions occurring only in a subset of cells in the investigated organ, as it is discussed below for the GFPC and 6MN X-ChIP experiments in *Arabidopsis*.

4.2.3 DEF/GLO interacts with *DEFH125* promoter in petals and stamens

DEFH125 is a pollen-specific MADS box gene that could be repressed by DEF/GLO, because it shows an expression domain complementary to the DEF protein (Zachgo et al., 1995; Zachgo et al., 1997). Preliminary X-ChIP data suggested an interaction between DEF/GLO and the promoter of *DEFH125* (Müller, 2004). Here, new extended interaction studies, by applying the new procedure for X-ChIP data analysis, confirmed the interaction.

A t Student test supported the enrichment of a *DEFH125* promoter region, including the CArG box 2, with respect to the background control located about 1000bp upstream, in wild type samples.

The regions flanking the CArG box 2 were still enriched with respect to the background, but significantly less than those spanning the binding site. The bell shaped curve extrapolated from these data is not perfectly symmetrical (Figure 11): the tail on the left hand side is longer than that on the right, close to the translation starting codon. In fact, the enrichment of the region 5 is not significantly higher than the background, setting the threshold at 5%. This could be due to an unequal DNA shearing by sonication, which can be caused by a different chromatin conformation.

A flat line could be extrapolated from the mutant sample data: all the value averages are not significantly divergent from each other, and could be part of the same population.

The *DEFH125* locus is inactive, hence probably free from transcription factors, apart from DEF/GLO. In fact, when *def-gli* mutant material was used, the serum crossreactivity registered for the *GLO* promoter was not detected and a flat line could be extrapolated.

The regulation of MADS box gene expression by the action of other MADS box factors has already been described for other cases: DEF/GLO and AP3/PI auto-activate their own transcription and AP1 activates AP3 (Lamb et al., 2002). The repression of *DEFH125* by DEF/GLO would be the first case of negative regulation in this MADS box regulative network.

One replicate of the experiment was carried out with an anti-GLO serum on wild type petals stage 3 and *glo* mutant flowers. A bell shaped curve could be extrapolated from wild type data and not from *glo* mutant data (not shown). This indicates, for the

first time, that DEF/GLO binds the DNA as a heterodimer *in vivo*. However, these data do not demonstrate that the DEF/GLO can repress the transcription of *DEFH125*. More evidence to support this topic can be collected by measuring the *DEFH125* expression in an inducible system, such as *def-101* (Zachgo et al*.,* 1995).

The closest *Arabidopsis* homologs of DEFH125 at the amino acidic level are AGL21 and AGL17, two MADS box proteins known to be expressed in roots (Burgeff et al., 2002). The Affymetrix data revealed that these two genes are also up-regulated in pollen, although for *AGL17* only weakly above background.

Therefore, AGL21 would be the best candidate for a functional homolog of DEFH125. It would be worth investigating if it is a target gene of AP3 by X-ChIP experiments in *Arabidopsis*.

As discussed above, the serum crossreactivity could hinder a demonstration of DEF/GLO-*GLO* promoter interaction by X-ChIP. No crossreactivity was reported for *DEFH125*, suggesting that no other MADS box transcription factor interacts with the *DEFH125* promoter.

Moreover, the *DEFH125* transcription is repressed by DEF/GLO direct interaction throughout petals and stamens. Hence no dilution of the specifically immunoprecipitated DNA fragments, as mentioned for the *EXTENSIN* promoter, would occur.

These two points would suggest that the use of the X-ChIP technique is suitable only for the investigation of genes repressed by the protein under investigation throughout the analyzed tissue.

4.2.4 X-ChIP experiments in *Arabidopsis*

X-ChIP experiments were performed in *Arabidopsis* to study the interaction of AP3 with putative target sequences. Two different systems were used: (1) transgenic lines where *DEF* complemented the *ap3* mutant and a polyclonal serum against DEF could be used, (2) *ap3-1* complemented transgenic lines where the AP3 protein was tagged with different peptide sequences against which commercially available antibodies could be employed.

Two putative target genes were investigated with both methods: the *AP3* gene itself, that was postulated to be bound by the AP3 protein to maintain its own expression

(Hill et al., 1998), and *NAP*, a gene that plays a role in the transition from the cell division to the cell expansion phase of growth of petals. *NAP* was proposed to be a direct target gene of AP3/PI by Sablowski et al. (1998).

The interaction between AP3 (or DEF) and the putative target DNA could not be confirmed by these X-ChIP experiments. In case of *NAP* genomic locus investigated with the *ap3-1*TDEF system, a bell shaped curve could be extrapolated from data collected from both the mutant (*ap3-1*) and the wild type-like system (*ap3-1*TDEF).

This case is analogous to the *GLOBOSA* one: the anti-DEF serum could crossreact with other factors interacting with the promoter of *NAP* in the *ap3-1* mutant flowers as well.

The second system, i.e. the *ap3-1* mutant plants complemented with tagged versions of AP3, was developed to circumvent this bottleneck, taking advantage of commercially available and immunopurified antibodies risen against peptides, such as the c-MYC tag or GFP, that do not share homology with transcription factors.

The full length coding sequence of *AP3* was expressed in *ap3* mutant plants as a N- and C- terminal fusion to five different tags: three times c-MYC (3MYC), six times c-MYC (6MYC), GFP, three times HA (3HA) and a Tandem Affinity Purification tag (TAP). 3MYC is the tag that interferes least with the AP3 activity, but only when expressed as a fusion to the C-terminus. This reveals that the presence of an artificial peptide at the N- terminus of a MADS box protein can disturb its function, probably interfering with the DNA binding activity, mediated by the N-terminal MADS domain. The same was observed for GFP. 6MYC is slightly larger than 3MYC but seems to interfere with the AP3 protein, since only one plant out of 20 showed a rescued phenotype. It was thus surprising to find that this one plant is an AP3 N-terminal fusion. The 3HA and TAP tags seem to inhibit the activity of AP3, since none of the 34 and 20 respectively transgenic plants showed a rescue of the *ap3* phenotype. As a conclusion, it seems that the amino acid composition and the position of the tag, rather than its size, can influence the protein activity (that is less inhibited with GFP than 3HA), although the size has a certain impact (3MYC is less invasive than 6MYC).

X-ChIP experiments using GFPC and 6MN transgenic plants and polyclonal antibodies against GFP or monoclonal anti c-MYC antibody gave a similar result: no region was particularly enriched.

This case is different from that reported for *GLO* or *ap3-1*TDEF and the failure to detect a binding site for AP3 requires a different explanation.

The easiest explanation would be that none of the investigated regions are actually bound by AP3/PI *in vivo*, although *in vitro* experiments would support the interaction of the heterodimer with at least two CArG boxes of the *AP3* promoter (Tilly et al., 1998; Hill et al., 1998).

On the other hand, the ability of the tagged version of AP3 to bind DNA is supported by the rescue of the mutant phenotype that demonstrates the functionality of the tagged AP3, and by the fact that the amount of IPed DNA from 6MN or GFPC samples is higher than from *ap3-1* mutant plants (not shown).

These two observations indicate that:

1) the tagged AP3 can interact with the DNA

2) the employed antibody is able to interact specifically with the tag, and much less with other factors.

Nevertheless the “6MN or GFPC vs. mutant” enrichment concerns any DNA sequence, so it can be only attributed to an increased background level.

This phenomenon, also observed in *Antirrhinum* and in *ap3-1*TDEF plants, could be explained with the PFA-induced protein-protein cross-links (Orlando et al., 1997) that could facilitate the connection of epitopes with any genomic sequence.

Whereas in the case of *NAP* in *ap3-1*TDEF some regions of the promoter are enriched with respect to the background control, both in *ap3-1* and *ap3-1*TDEF, it seems that for the tagged AP3 lines the background is too high. Furthermore, the chromatin superstructure could not allow the antibody to access the tagged AP3, when located on the investigated DNA regions.

Finally, it should be considered that the expression domain of the investigated genes, *NAP* and *AP3*, hence the tissues where AP3/PI is supposed to bind their promoter, is restricted to petals and stamens, that represent only a portion of the inflorescences harvested for the X-ChIP experiments. The specifically immunoprecipitated sequences are consequently diluted, similarly to what has been described for the *EXTENSIN* experiments.

The conclusion one can draw from this scenario is that a specific tagged AP3-DNA interaction is very likely to occur *in vivo*, but it cannot be detected because the ratio of specifically immunoprecipitated molecules/background is too low.

The experiments conducted in *Arabidopsis* confirmed two major bottlenecks of the X-ChIP technique, already highlighted for the *GLO* and *EXTENSIN* experiments in *Antirrhinum*: the crossreactivity of the used serum can prevent the demonstration of the interaction of a protein with an active genomic locus. Furthermore, as the interaction occurs only in a subset of cells of the considered sample, it might not be detectable because of a high dilution of the specifically immunoprecipitated DNA fragments.

4.2.5 Conclusions

In this work, *DEFH125* represents the only success to prove the protein-DNA interaction by X-ChIP, whereas for the other genes no conclusion could be drawn. As discussed, the causes of this failure could be attributed to a low “specifically immunoprecipitated molecules”/”background” ratio (*EXTENSIN* and GFPC and 6MN system) or to cross-reactivity of the used serum (*DEFICIENS*, *GLOBOSA*, *ap3-1*TDEF system).

These results were achieved by applying a new approach that allows the reliable identification of *in vivo* protein-DNA interactions by X-ChIP experiments. Setting a reliable procedure for data analysis is absolutely required in the process of improving the X-ChIP protocol, from tissue fixation to DNA immunoprecipitation, because it allows discerning false positive and real positive interactions.

Once a proper way of evaluating the results has been established, many parameters can be changed in order to enhance the performance of the technique, for instance by decreasing the background to detect protein-DNA interactions. These parameters can be grouped into five categories:

1) Fixation agent: only PFA was used so far because it induces fully reversible crosslinks. PFA induces protein-protein crosslinks as well, and this could contribute to increasing the background. Many other crosslinking agents, e.g. cisplatin (Spencer and Davie, 2002), are commercially available, although not as extensively used as PFA (Spencer et al*.,* 2003).

2) Fixation condition: for *DEFH125* it was observed that by shortening the PFA incubation time, the specifically immunoprecipitated DNA was reduced as well (not shown). On the other hand, an overfixation increases the background, showing that

the fixation is a crucial step in the X-ChIP protocol. The optimal incubation time and PFA concentration for *Antirrhinum* and for *Arabidopsis* was established according to Orlando et al. (1997). Briefly, the PFA-induced crosslinks prevent DNA shearing by sonication. By comparing on Et/Br-Agarose gels DNA extracted from samples differently treated and sonicated, the optimal conditions that grant a minimum fixation were determined.

However, it is possible that fixation conditions, incubation time, PFA concentration and incubation buffer composition can still be optimized to improve the X-ChIP protocol.

3) Serum: in the described experiments polyclonal and monoclonal sera raised against DEF or tags have been used. As discussed above, the failure of demonstrating a protein-DNA interaction could be partially attributed to a low specificity of the sera. If so, other tags could be used, or the anti-DEF serum could be immunopurified by running through a column where the DEF protein is fixed to a matrix. Alternatively, other kinds of affinity purification could be tried: replacing the tag on AP3 fusion proteins with a biotinylation signal and performing the precipitation with streptavidin coupled beads could be an option. It has been reported that, due to the high biotin-streptavidin affinity, the precipitation condition can be more stringent, allowing to reduce the background (Viens et al. 2004).

4) Immunoprecipitation reagents: the immunoprecipitation was carried out as previously described (Müller, 2004; Wang et al*.,* 2002). Improvements to this protocol might still be possible. For instance, the RIPA buffer, used in the sepharose beads-mediated immunoprecipitation and in the subsequent washing steps, could be replaced with another one that makes conditions more stringent (Spencer et al., 2003).

5) Double immunoprecipitation: a tandem double immunoprecipitation (with anti-DEF and anti-GLO sera) showed to be successful in increasing the ratio of putative binding site/background control, although the test was this far only performed on the DEF genomic locus (that was not confirmed as a target of DEF/GLO) and a "*def-gli* mutant" control was not included (2.2, results).

Including an additional immunoprecipitation step in the X-ChIP protocol could reduce the background and circumvent serum-related cross reactivity. However, it should be considered that the DNA rescued after the double immunoprecipitation is much less

than after a single one (not shown), and the subsequent SQ-PCR products could be difficult to quantify and reproduce.

6) Circumventing the dilution of specific immunoprecipitated DNA fragments: *DEFH125* is thought to be bound by DEF/GLO throughout the investigated tissue, hence no dilution problem occurs as hypothesized for EXTENSIN and GFPC and 6MN. To circumvent a similar bottleneck in the investigation of target genes of LEAFY (LFY), a floral regulator normally expressed in a very narrow domain of the flower meristem, Dilusha et al. (2004) set up an inducible system where LFY was expressed as a fusion to the Glucocorticoid Receptor (GR) under the control of the CaMV 35S promoter. The fusion protein, expressed throughout the plant, is translocated into the nucleus only upon treatment with dexamethasone. X-ChIP experiments were carried out with an anti-LFY serum and the target genes enrichment in dexamethasone- and mock-treated tissues was tested.

This approach could lead to artificial protein/DNA interactions as the ectopically expressed protein is not investigated in its natural context. For this reason a similar approach would not be advisable to the AP3/PI target gene studies: the AP3/PI heterodimer regulates different sets of target genes to give rise to very different organs like petals or stamens. Hence its activity is strictly whorl-specific and requires the presence of cofactors, although carrying out organ-specific X-ChIP experiments in *Arabidopsis*, as performed with *Antirrhinum*, might be problematic because of difficulties in harvesting material.

4.3 Class B factors in yeast studies

Floral homeotic MADS box transcription factors were postulated to interact with their DNA target sequences as quartets, i.e. as complexes of four proteins. This theory comes from the observation that the DEF/GLO DNA binding capacity is enhanced by a third protein (SQUA), suggested to participate in the complex as a homodimer. Moreover, AP3/PI can interact in yeast with other MADS box proteins, like SEP3 or AP1 (Egea-Cortines et al., 1999; Honma et al., 2001).

In *Arabidopsis*, the constitutive expression of AP3 and PI together with SEP3 or AP1, or both, activates the transcription of a GUS reporter gene cloned downstream of the *AP3* promoter in tissues where *AP3* normally is not expressed (Honma et al., 2001). The constitutive and simultaneous expression of AP3-PI-SEP3-SEP2-AP1 is sufficient to transform a vegetative organ, e.g. leaf, towards a petaloid structure (Pelaz et al., 2001). Nevertheless, the *AP3* transcription is normally activated in *sep1 sep2 sep3* triple mutants and is still detected in second whorl organs in *ap1* and *pi* mutants, but not in *ap3* mutants (Hill et al., 1998; Jack et al., 1992; Jack et al., 1994; Pelaz et al., 2000). Combining these data one could conclude that the combined action of AP3/PI/SEP3/AP1 is sufficient to activate the transcription of *AP3* and target genes in petals, but PI, SEP3 and AP1 are not absolutely required for the initiation and maintenance of the *AP3* transcription in petals.

These considerations, together with the inability of AP3 to bind to DNA *in vitro* as a homodimer (Hill et al., 1998), suggest that AP3 interacts also with other factors, at least to bind its own promoter and maintain its own expression in petals.

In an effort to isolate more AP3 and AP3/PI cofactors, a yeast three hybrid system was set up: AP3 was coexpressed as a fusion to the GAL4 BD, together with PI and proteins encoded by a cDNA library, the latter expressed as fusion to the GAL4 AD. This system allowed the identification of AP3 interactors and ternary interaction factors that require the simultaneous presence of AP3 and PI.

Moreover, a new system that combines the one-, two- and three hybrid technologies was set up in an attempt to study the interaction of AP3-PI-SEP3-AP1 with the *AP3* promoter in yeast.

4.3.1 AP3/PI three-hybrid screening

Four new interactors of AP3 were isolated in a three-hybrid screening, using AP3 and PI as baits: At1g04190, At1g79150, At1g19100 and At1g72740. These four proteins do not require the coexpression of PI to form complexes with AP3.

At1g04190, *At1g79150* and *At1g19100* share a very similar expression profile. The three proteins are supposed to have a transport or kinase activity. They could be involved in the posttranslational modifications and transportation of AP3, as it was described for AGAMOUS-LIKE 24 (AGL24) protein, which is translocated to the nucleus upon phosphorylation (Fujita et al., 2003).

The fourth interactor, At1g72740, is a member of the MYB transcription factor family and is the first interacting non MADS box transcription factor isolated for AP3. At1g72740 from *Arabidopsis* is the closest homolog of *Pc*MYB1 from parsley that has been shown to interact *in vitro* with the promoter of *Chalcone synthase* (*CHS*) (Feldbrügge et al., 1997). CHS is an enzyme that is present in a single copy in *Arabidopsis*, and is thought to be involved in the synthesis of secondary metabolites such as flavonoids or anthocyanins (Hahlbrock and Scheel, 1989). The expression pattern of *Arabidopsis CHS* (*AtCHS*) resembles that of AP3 in developing flowers; the expression of *AtCHS* is stronger in young petals than in the other floral organs.

It is tempting to speculate that *AtCHS* is positively regulated by AP3 through an interaction with At1g72740, although the role of CHS in flower development is not clear. In fact, a mutation in *AtCHS* abolishes the production of flavonoids in *Arabidopsis*, but neither affects petal color, as observed in *Antirrhinum* (Coen and Carpenter, 1988), nor the male fertility, as observed in *Zea mays* (Burbulis et al., 1996).

Investigation of the *AtCHS* promoter revealed a MYB recognition element (MRE) motif, conserved in species closely related to *Arabidopsis.* No CArG boxes could be identified (Koch et al., 2001). However, it would be interesting to investigate by X-ChIP the ability of AP3 to bind the promoter of *AtCHS* in *Arabidopsis*, or of DEF to bind the promoter of *NIVEA*, the *Antirrhinum* homolog of *CHS*.

4.3.2 The AP3/PI/AP1/SEP3-DNA interaction cannot be reproduced in yeast

In an attempt to study in yeast the AP3/PI/AP1/SEP3-*AP3* promoter interaction, constructs expressing AP3, PI, SEP3 and AP1 were prepared. These four proteins have been expressed in yeast in various combinations and together with the reporter vector, where two variants of the *AP3* promoter (200 and 800bp long promoter fragments) were cloned upstream of the HIS3 reporter gene, in a scenario very similar to the *in planta* experiment reported by Honma et al. (2001).

This pilot experiment addressed the question if MADS box protein interaction with DNA can be studied in an environment closer to the *in vivo* scenario than the classical two or three-hybrid systems. In fact, the DNA harboring function, conducted by the GAL4 BD in the classical two- or three hybrid systems, would be carried out by the MADS proteins themselves, that own an endogenous DNA binding domain (the MADS box). Therefore UAS, the DNA element interacting with GAL4 BD, was replaced with CArG boxes contained in the *AP3* promoter*,* that could participate in the protein interaction and dimer stabilization (Tilly et al*.,* 1998; Hill et al*.,* 1998). Moreover, Egea-Cortines et al*.* (1999) and Theissen and Saedler (2001) hypothesized that the MADS quartets could interact more strongly, inducing a DNA loop, with promoters like the one of *AP3* that contains two CArG boxes (model schematized in Figure 3).

SEP3 has a C-terminal activation domain shown to be functional in yeast, so it was not expressed as a fusion to the GAL4 AD (Honma et al., 2001). Other proteins, like AP3 or PI, that do not own an activation domain, were expressed as a C- or N-terminal fusion to the GAL4 AD.

An identification of AP3/PI ternary cofactors that need both proteins as well as the interaction with DNA would be possible. Then amino acid substitution experiments could be carried out with newly identified interactors to figure out which residues are important for DNA interaction or dimer formation.

Moreover, the system could be employed for screening a genomic DNA library, cloned upstream of the HIS3 reporter gene in the ycphis3bs vector, in order to isolate DNA sequences able to bind MADS protein dimers or tetramers. A genomic library generated in an X-ChIP experiment, hence already enriched in AP3/PI or DEF/GLO

target sequences, could be screened to isolate sequences bound by the dimer/tetramer *in vivo*.

No yeast growth was registered and no interaction could be detected, in spite of expressing the MADS box proteins in their *in vivo* conformation, by omitting the GAL4 AD or artificial NLS, that could cause protein misfolding. The system relies on the natural ability of AP3/PI to be targeted to the nucleus, and of SEP3 to activate transcription in yeast. Since the functionality of the AP3/PI NLS was shown *in planta* only (McGonigle et al., 1996), an artificial NLS was fused to the AP3 and PI C-terminus, where it is less likely to interfere with the DNA binding activity.

A similar experiment was carried out with DEF, GLO and SQUA, expressed in their *in vivo* conformation or as a fusion to the GAL4 AD. The output was exactly the same, no yeast growth was observed.

All the yeast assays here reported were performed at 28°C, the yeast optimal temperature, and at 22°C, the optimal temperature for *Arabidopsis* MADS box protein interaction in yeast, as suggested by Honma et al. (2001).

It has already been reported in the literature that the interaction of AP3/PI and partners requires special conditions: Honma et al. (2001) failed to detect any interaction between AP3/PI and AP1 or SEP3 at 28°C, and Yang et al. (2003) could not observe an interaction between AP3 and PI, unless the MADS domain was removed from both proteins.

A possible explanation for the failure in reproducing the *in vitro* and *in vivo* observations could be the inadequacy of the yeast environment; this could concern an incorrect folding of the protein, differences in pH values or electrolyte concentration, or the presence of competitors. The conclusion that one could draw from this experiment is that the yeast environment is not suitable for the study of plant MADS box protein interactions.

However, the yeast system was successfully used to isolate well characterized *in vivo* functional partners of AP3 and AP3/PI, such as PI, SEP3 and AP1.

Considering these data together, one could conclude that the yeast system, although eukaryotic, is quite different from *Arabidopsis*, hence could be of limited use to study AP3/PI interactions.

4.4 ROXY interactors

Class B homeotic genes control the development of organ primordia that are set on the floral meristem by another group of genes, including *ROXY1*. ROXY1 is the only plant glutaredoxin (GRX) so far characterized and is involved in second whorl organ initiation and development: *roxy1* flowers form less petals that occasionally show an altered curvature (Xing et al., 2005).

GRXs are small proteins involved in redox reactions. GRXs can influence the structure of their target proteins by reducing disulphide bridges or oxidizing sulfhydryl groups. The addition or the removal of a glutathione, a process known as glutathionilation or deglutathionylation, can also be mediated by GRXs. GRXs and their target proteins from animals, yeast and bacteria, have been described to be involved in many pathways and functions, like stress response, protein conformation and cytoskeleton. (Rouhier et al., 2004; Lemaire, 2004).

In *Arabidopsis*, a few GRXs were studied, that are thought to interact with H^+/Ca^{++} transporters or with enzymes like aldolase or isomerase, reflecting the broad variety of target proteins already found in other organisms (Rouhier et al., 2004).

ROXY1 and ROXY2 were used as baits in two yeast two-hybrid screenings, to identify proteins that might be modified by the GRX activity. About 35 clones, encoding for a broad variety of functionally different proteins, were isolated. Only interactors that might be functionally linked to ROXY1 were considered (TCP3, TCP4, TCP14, TCP21, SnRK, TGA3, RBE and AmTCP2) for further full length tests to confirm the interaction.

The TGA3 transcription factor and three members of the TCP transcription factor family, TCP3, TCP4 and TCP21, were confirmed to represent positive interactors in yeast. ROXY1 and ROXY2 share the same affinity for these four interactors, suggesting a certain redundancy in their function.

Posttranslational modifications, like phosphorylation, methylation and acetylation, have been shown to regulate the activity of transcription factors that affect organ development (Molkentin et al., 1996; Ehsan et al., 2004). TGA3 and TCP transcription factors might be regulated by ROXY1 during petal initiation.

TGA3 belongs to the TGA transcription factor family. Some TGA transcription factors, such as TGA2.2, were shown to interact with glutaredoxin. Furthermore, TGA1 changes its DNA-binding affinity in a redox state dependent manner (Després et al., 2003; Mrief Ayed, 2004). TGA3 and other TGA proteins have been demonstrated to be involved in pathogen response and Ca^{++} mediated signalling pathways (Zhou et al*.,* 2000; Szymanski et al*.,* 1996). The finding that a TGA transcription factor might be involved in development regulation is new.

Some members of the TCP family were described to play a role in cell proliferation (Cubas et al*.*, 1999). *TCP3* and *TCP4* contain a nucleotide sequence outside the TCP domain that is conserved among members of the CIN group, and which is a target of a miRNA. The ectopic expression of this miRNA causes a reduction in the *TCP3* and *TCP4* transcripts, resulting in leaf cell proliferation misregulation that causes a phenotype that resembles *cin* in *Antirrhinum* (Palatnik et al*.*, 2003; Nath et al*.,* 2003). These data strengthen the importance of TCP proteins in the control of cell proliferation.

The expression profiles of *ROXY1* and *TCP* genes were investigated by analysis of the Affymetrix data, that revealed a coexpression of these genes in the shoot apical meristem (SAM), supporting an *in vivo* interaction of these proteins. *In situ* analysis reported by Xing et al*.* (2005) revealed that *ROXY1* expression is not limited to the SAM, as one could deduce form the microarray data, but extends into floral meristems, petal and stamen primordia at early stages of development, and into vascular bundles and ovules in older flowers.

This distinctive expression pattern of *ROXY1* was not revealed by microarray data, as it is restricted to a few cells only.

The ROXY1 activity in petal initiation and development could be linked to its expression in flower meristem, petal primordia and developing petals, rather than to its expression in the SAM. Therefore, an *in situ* hybridization analysis of *TCP3*, *TCP4* and *TCP21* expression was conducted to investigate if overlapping expression patterns support a functional interaction between ROXY1 and these TCP proteins in petal initiation and development.

The *in situ* analysis of *TCP3*, *TCP4* and *TCP21* revealed indeed overlapping expression domains with *ROXY1*: the transcripts of these three *TCP* genes and of *TCP14* were detected in the inflorescence and floral meristems and in organ

primordia. At later stages of floral development, the expression pattern became more diverse and distinct: *TCP3* is expressed only in petal tips, confirming what has already been reported (Cubas et al., 1999); *TCP4* is expressed in petals, sepals and ovules; *TCP21* remains ubiquitously expressed, and *TCP14* expression becomes restricted to the abaxial sepals and ovules.

Considering these expression data together, *TCP3* shows the most similar expression profile to that of *ROXY1*.

With respect to the known TCP function, another aspect of the TCP14 expression is interesting. In *Arabidopsis*, the abaxial sepal develops more rapidly and grows over the adaxial ones (Figure 26). At older floral stages, the expression domain of *TCP14* is restricted to the abaxial sepals. This scenario is similar to the expression of *CYC* in *Antirrhinum* (Luo et al., 1996; Eckardt, 2001), which is specific for the dorsal petals where it is assumed to promote outgrowth. The ventral petal, lacking *CYC* expression, remains smaller. *TCP14* could be involved, like *CYC*, in organ specific cell elongation or proliferation.

It was reported that GRXs, used as bait in yeast two-hybrid screenings, can interact with a number of prey proteins (Yamazaki et al., 2004; Lemaire et al., 2004), corroborating the high number and broad variety of interactors found for ROXY1 and ROXY2.

Being the ROXY1 in yeast interactors so many and different, the physical affinity of ROXY1 for them might not be very specific. *In vivo* interaction specificity could be restricted to the coexpressed interactors, and could be improved by the participation of third factors.

Other than the overlapping expression, a functional link supports the *in vivo* interaction between the isolated TCP proteins and ROXY1. In fact, the first is involved in cell proliferation and the second is responsible for cell proliferation in petals.

The fact that both ROXY1 and ROXY2 can interact with all the TCP proteins tested, even with one from *Antirrhinum* (although ROXY2 only very weakly), and the fact that every clone isolated from the two-hybrid screening, although incomplete, contains the TCP domain, indicates that the specificity of ROXY1 and ROXY2 toward TCP proteins might not be restricted to TCP3, TCP4 and TCP21. It is tempting to speculate that the whole TCP class could be posttranslationally modified by ROXY1.

Future studies can now focus on demonstrating the involvement of ROXY1 in posttranslational modifications of TCP proteins. To investigate the capacity of thioredoxin to bind to target proteins and to change their redox state *in vitro*, Motohashi et al. (2001) used a chromatography system where the thioredoxin was immobilized to a resin. Yano et al. (2001) used subtraction display of the proteins in which the cysteines were labelled with a fluorescent dye before and after the incubation with the TRX, determining the differences by two-dimensional SDS-PAGE. In order to detect S-glutathionylated proteins, Fratelli et al. (2002) proposed an *in vivo* approach in human cell lines: cysteines labelled with radioactive sulphur (^{35}S) were introduced into the cell under conditions of protein synthesis inhibition; proteins were then extracted from the cells, separated by gel electrophoresis and differences were detected by autoradiography and sequence analysis.

These methods can be adapted to deliver proof for an *in vivo* interaction of ROXY1 with the target proteins identified in this work. Moreover, the study of *Arabidopsis* mutants for these genes could further support their functional interaction *in vivo.*

4.5 Conclusions

In the work presented here, a molecular analysis of petal initiation and development has been conducted.

The X-ChIP technology was improved and applied to the study of class B target genes. The X-ChIP technique is currently the only protocol that allows demonstrating a protein-DNA interaction *in vivo*, and was successfully used in the study of DEF/GLO-*DEFH125* promoter interaction. The failure to demonstrate a protein-DNA interaction for the other tested genes revealed that the technique might not be suitable under certain circumstances.

Serum crossreactivity and a high level of background seem to be the two main limitations of this technique, limiting the use of the technique to genes, like *DEFH125*, that are negatively regulated throughout the investigated organ.

The isolation of new target genes could be hindered by two main bottlenecks: a high background that renders the isolation of a sequence bound by DEF unlikely and the

fact that DNA interaction does not necessarily imply also a transcriptional control function.
However, a tandem double immunoprecipitation with anti-DEF and anti-GLO sera could help in reducing the background and improving the experiment output.

A MYB protein (At1g72740), putative activator of the *Chalcone synthase* (*CHS*), was found to be a novel interactor of AP3. The high expression level of *CHS* in petals and developing flowers, and the fact that the homologs of *CHS* in *Zea mays* and in *Antirrhinum* are required for male fertility and flower pigment biosynthesis, respectively, suggests that *CHS* might be a target gene of AP3 and At1g72740. This observation could not be corroborated in *Arabidopsis* by the *chs* mutant phenotype that does not show any alteration in flower development or pigmentation. However, it could be worth investigating if *CHS* is controlled by AP3 in *Arabidopsis*, or by DEF in *Antirrhinum*.

Three members of the TCP family were found to interact with the glutaredoxin ROXY1. The *TCP* genes encode transcription factors that regulate cell proliferation. ROXY1 could modify TCP proteins by the addition/removal of a glutathione or oxidation/reduction of intermolecular disulphide bridges. The posttranslational modification could result in the activation or repression of the TCP function, hence in cell proliferation control. A lack of TCP regulation could lead to petal abortion and aberrant development, as observed in the *roxy1* phenotype. Further analysis of *TCP* mutant phenotypes will allow to investigate the role of TCPs in petal initiation and development.

5. Summary-Zusammenfassung

5.1 Summary

Flowers of eudicots like *Antirrhinum* and *Arabidopsis* are composed of four concentric whorls, where four different kinds of organs develop: sepal and petals in the two outermost whorls and the sexual organs, stamens and carpels, in the centre of the flower.

Organ development is determined by homeotic genes that interact according to the ABCDE model. The homeotic B class genes, represented by *DEFICIENS* (*DEF*) and *GLOBOSA* (*GLO*) in *Antirrhinum* and *APETALA3* (*AP3*) and *PISTILLATA* (*PI*) in *Arabidopsis*, participate together with class A and E factors in regulating petals development and together with class C and E in stamens development.

In the work here presented, class B target genes are investigated in *Antirrhinum* and *Arabidopsis* by Crosslinked Chromatin ImmunoPrecipitation (X-ChIP) experiments, to prove the direct protein-DNA interaction by an immunoprecipitation.

In this study a new approach is presented and discussed for the X-ChIP data analysis that includes more controls with respect to the standard procedure reported in literature, and that allows the discrimination of false positive results. This new approach showed that the interaction of DEF protein with the promoter of the putative targets *GLOBOSA*, *DEFICIENS* and *EXTENSIN* is not demonstrable at these conditions and revealed its interaction with the promoter of *DEFH125*.

Protein-protein and protein-DNA interactions that involve Class B factors have been studied in yeast: new interactors of AP3 and PI were isolated by a three-hybrid screening.

Class B transcription factors determine the fate of petal primordia, but not their initiation, which is controlled by other genes, like *ROXY1*, which belongs to the plant glutaredoxin (GRX) family. *roxy1* mutant phenotype presents a reduced number of petals and abnormalities during further petal development. GRXs are small proteins involved in posttranslational modifications of target proteins via redox reactions. In order to shed light on its activity, ROXY1 was used as bait in a two-hybrid screening. Among many isolated clones, the transcription factors TCP3, TCP4 and TCP21 were

chosen for a further investigation. TCP proteins have been shown to participate in the control of cell proliferation and can be functionally linked to ROXY1 because, at early stages of *roxy1* developing flower, misregulation of cell proliferation leads to phenotypic abnormalities. In order to support an *in vivo* interaction, *in situ* hybridizations were conducted, revealing that the expression domains of *TCP3*, *TCP4* and *TCP21* overlap with that of *ROXY1* at early stages of floral development.

5.2 Zusammenfassung

Blüten eudikotyler Pflanzen wie *Arabidopsis* und *Antirrhinum* bestehen aus vier konzentrischen Wirteln, die verschiedene Organe bilden: Sepalen und Petalen in den beiden äusseren Wirteln, und die sexuellen Organe - Stamen und Karpelle - im Zentrum der Blüte. Die Organentwicklung wird gesteuert durch homöotische Gene, die nach dem ABCDE-Modell miteinander interagieren. Die homöotischen B-Klasse Gene, die in *Antirrhinum* durch *DEFICIENS* (*DEF*) und *GLOBOSA* (*GLO*), und in *Arabidopsis* durch *APETALA3* (*AP3*) und *PISTILLATA* (*PI*) repräsentiert werden, wirken zusammen mit Klasse-A und -E Genen in der Petalenentwicklung, und mit den Klasse-E und -C Genen in der Stamenentwicklung.

In der vorliegenden Arbeit wurden Zielgene der Klasse-B Faktoren in *Antirrhinum* und *Arabidopsis* mit der Crosslinked Chromatin ImmunoPrecipitation (X-ChIP)-Technik untersucht, um eine direkte Protein-DNA-Interaktion durch Immunofällung nachzuweisen.

Es wird hier eine neue Herangehensweise an die X-ChIP-Datenanalyse präsentiert und diskutiert, die im Vergleich mit Standardprotokollen aus der Literatur mehr Kontrollen beinhaltet und ein Erkennen falscher Positivresultate erlaubt. Dieser neue Ansatz zeigte, dass die Interaktion des DEF-Proteins mit den Promotoren der vermeintlichen Zielgene *GLOBOSA*, *DEFICIENS* und *EXTENSIN* unter den gegebenen Konditionen nicht demonstriert werden kann, dafür aber eine Interaktion von DEF mit dem Promoter von *DEFH125*.

Interaktionen zwischen Proteinen und Protein und DNA an denen B-Klasse Gene beteiligt sind, sind bisher in Hefe untersucht worden. Hierbei konnten neue Interaktoren von AP3 und PI durch Hefe-2-Hybrid Screens isoliert werden.

Transkriptionsfaktoren der Klasse B determinieren das Schicksal von Organprimordien, nicht aber deren Initiierung, welche durch andere Gene, wie z.B. *ROXY1* kontrolliert wird. *ROXY1* gehört zur Familie der pflanzlichen Glutaredoxinen (*GRX*). Individuen, die für *ROXY1* mutant sind, zeigen eine Reduktion der Petalenanzahl und Abnormalitäten während der weiteren Petalenentwicklung.

Glutaredoxine sind kleine Proteine, die die posttranslationale Modifikation ihrer Zielgene über Redoxreaktionen bewirken. Um die Aktivität von ROXY1 näher zu untersuchen, wurde das Protein als ‚Köder' in einem Hefe-2-Hybrid Screen verwendet. Unter vielen isolierten Klonen wurden die Transkriptionsfaktoren *TCP3, TCP4* und *TCP21* zu weiteren Studien ausgewählt. TCP-Proteine wirken an der Kontrolle von Zellproliferation mit und können funktionell mit ROXY1 in Zusammenhang gebracht werden, da eine Misregulation der Zellteilung in *roxy1*-Mutanten in frühen Stadien der Blütenentwicklung zu phänotypischen Abnormalitäten führt. Um die beschriebene *in vivo* Interaktion zu unterstützen, wurden *in situ* Hybridisierungen durchgeführt, die zeigten, dass in frühen Stadien der Blütenentwicklung die Expressionsdomänen von *TCP3, TCP4* und *TCP21* mit der von *ROXY1* überlappen.

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7. Appendix

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7.2 Eidesstattliche Erklärung

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

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