AmGRAS, a GAI and RGA like protein interacts with ROSINA (RSI), a transcription factor which may control expression of the B- function gene *DEFICIENS*

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To my Father

Contents

Abbreviations

3-AT	3-Amino-1,2,4-Triazole
A_X	Absorbance at X nm
A. thaliana	*Arabidopsis thaliana*
A .majus	*Antirrhinum majus*
APS	Ammonium Persulphate
bp	Base pair
BSA	Bovine Serum Albumin
cDNA	Complementary Deoxyribonucleic Acid
C-terminal	Carboxy terminal
DEPC	Diethyl pyrocarbonate
dCTP	2'-Deoxycytidine-5'- triphosphate
DIG	Digoxigenin
DMSO	Dimethyl sulfoxide
dNTP	Deoxynucleoside triphosphate
DTT	1,4-Dithiothreitol
EDTA	Ethylenediamine tetraacetic acid
EtOH	Ethanol
g	Gram
GST	Glutathione S-transferase
h	Hour
IPTG	Isopropyl-β-D-thiogalactopyranoside
kb	Kilobase
l	Liter
mg	Milligram
min	Minute
ml	Milliliter
mM	Millimolar
mRNA	messenger Ribonucleic acid
N-terminal	Amino terminal
Nr	Number
ONPG	2-Nitrophenyl-β-O-galactopyranoside
ORF	Open Reading Frame
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase Chain Reaction
PEG	Polyethylene glycol
RNase	Ribonuclease
Rpm	Revolutions per min of roter
RT	Room Temperature
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SDS	Sodium dodecyl sulfate
TEMED	N,N,N',N'-Tetramethylethylenediamine
Tris	Tris(hydroxymethyl)-amino-methane
µg	Microgram
µl	Microliter
V	Volt
WT	Wildtype
X-Gal	5-Bromo-4-Chloro-3-indolyl-β-D-galactopyranoside

Amino acids

A(Ala)	Alanine
C(Cys)	Cysteine
D(Asp)	Aspartic acid
E(Glu)	Glutamic acid
F(Phe)	Phenylalanine
G(Gly)	Glycine
H(His)	Histidine
I(Ile)	Isoleucine
K(Lys)	Lysine
L(Leu)	Leucine
M(Met)	Methionine
N(Asn)	Asparagine
P(Pro)	Proline
Q(Gln)	Glutamine
R(Arg)	Arginine
S(Ser)	Serine
T(Thr)	Threonine
V(Val)	Valine
W(Trp)	Tryptophan
Y(Tyr)	Tyrosine

1. Introduction

1.1 Flower development in angiosperms

Organ development in plants occurs largely post-embryonic (Steeves and Sussex, 1989). Plant embryos contain only a small fraction of the final body plan. Organs develop throughout the lifetime of the organism, emerging from the shoot and root meristems in response to different endogenous and environmental signals.

Flower formation in higher plants is a complex process influenced and controlled by genetic as well as environmental factors (Steeves and Sussex, 1989). The transition to flowering is a fundamental change in the life of a plant which means the change from vegetative to reproductive development. The transition occurs in shoot meristems, which are reprogrammed to produce inflorescences or flowers on receiving appropriate environmental and developmental signals. There are two types of transition. In the determinate type, the inflorescence meristem produces a terminal flower, whereas in the indeterminate type the inflorescence meristem gives rise to a number of floral meristems which develop into flowers.

Floral organs in dicotyledonous plants develop in four concentric rings (whorls). Sepal primordia arise first in the outermost whorl, around the periphery of the meristem dome. Petal primordia emerge next in alternate positions in the second whorl. Stamen (male organ) primordia appear in the third whorl, and finally, carpel (female organ) primordia are produced in the innermost whorl. In general, the structures of sepals and petals are displaying dorsoventral differences (there are differences in cell types on the upper and the lower sides) almost from the beginning, whereas this is not the case for stamens and carpels during early stages of development.

1.2 *Antirrhinum majus* as a model plant

Antirrhinum majus, a member of the Scrophulariaceae, is one of the well-studied model plants that contributed to the understanding of genetic and molecular processes of flower development.

During vegetative growth, *Antirrhinum* plants have pairs of leaves at each node. Each leaf pair is arranged in decussate phyllotaxy (successive leaf pairs being set at right angles to each other) with long internodes separating them. After the vegetative meristem has changed to the inflorescence meristem, much smaller leaves (bracts) are produced in a spiral arrangement. The bracts are separated by short internodes, and each has a floral meristem in its axil (Carpener *et al*., 1995). The flower is composed of five sepals, five petals, four stamens (male organs) and one stamenoid (aborted stamen), and two fused carpels (female organs) arranged in a whorled phyllotaxy. The basal parts of the petals are fused and form the corolla tube, while the higher parts form the upper (adaxial) and lower (abaxial) lobes. The flower of *Antirrhinum* is zygomorphic, which means that it has a vertical axis with bilateral symmetry.

As a model system for studying flower development, *Antirrhinum majus* has several advantages.

1. The transposon mutagenesis technique to generate new mutants has been established and transposable elements have been used as a tool for isolation of genes (transposon tagging) in *Antirrhinum majus* (Coen and Carpenter, 1986; Sommer *et al*., 1988).

2. Many developmental mutants have been isolated by various groups (Stubbe, 1966), and most of them are still available.

3. The plant can be vegetatively propagated, therefore allowing the rescue of sterile mutants.

4. The large flowers of *Antirrhinum* make crossing experiments easier than with *Arabidopsis* and facilitate collecting of sufficient material for molecular studies.

5. A good genetic map exists (Stubbe, 1966).

6. A transformation technique was established (Heidmann *et al*., 1998).

1.3 The classical ABC model of floral organ identity

During the past decade, there has been a great advance in our understanding of flower development. Floral organ identity is genetically controlled and can be described by the classical ABC model which is based on the genetic analysis of single, double and triple mutants.

The appearance of floral organs, their type, number and position is controlled by a set of regulatory genes (Meyerowitz *et al*., 1989; Schwarz-Sommer *et al*., 1990; Bowman *et al*., 1991; Coen, 1991). Mutations in these genes result in organs with morphologically altered phenotypes, mutant floral organs have lost their original wild type identities. The different classes of mutations known as 'homeotic' are particularly important and useful for understanding the genetic programs controlling development. Many floral developmental mutants with defects in the control of meristem identity and organ types are available and are studied in snapdragon (*Antirrhinum majus*) and thale cress (*Arabidopsis thaliana*).

Morphological and genetic analysis of the floral homeotic mutants in these plants led to the classical ABC model (Fig.1) which tries to explain how the specification of organ identity during flower development is controlled (Haughn and Sommerville, 1988; Schwarz-Sommer *et al*., 1990; Coen and Meyerowitz, 1991).

The ABC model proposes three function A, B and C which are active each in two adjacent whorls. The functions are established by the homeotic genes which can be grouped in three classes: A, B and C. In *Antirrhinum*, an A-function gene like in *Arabidopsis* has not been reported; B-function genes are *DEFICIENS* (*DEF*) and *GLOBOSA* (*GLO*). The C-function

gene is *PLENA* (*PLE*). In *Arabidopsis*, the A-function is established by both *APETALA1* (*AP1*) and *APETALA2* (*AP2*), the B-function by *APETALA3* (*AP3*) and *PISTILLATA* (*PI*), and the C-function by *AGAMOUS* (*AG*).

Class A genes function in whorls 1 and 2, class B in whorls 2 and 3, and class C in whorls 3 and 4. Expression of the class A genes alone determines sepal development, combinatorial action of class A and B genes or class B and C genes specifies petals or stamens, respectively, and expression of the class C genes alone results in carpel development. The activity of class A and class C genes are mutually antagonistic, so that A genes prevent expression of C genes in whorl 1 and 2, and C genes prevent expression of A genes in whorl 3 and 4. Mutants of class A genes have carpels in the first whorl instead of sepals. Class B mutants have sepals rather than petals in the second whorl and carpels rather than stamens in the third whorl. Class C mutants have petals instead of stamens in the third whorl and replacement of the carpels in the fourth whorl by sepals. Class C mutants are indeterminate, so there is a continued production of mutant floral organs inside the fourth whorl.

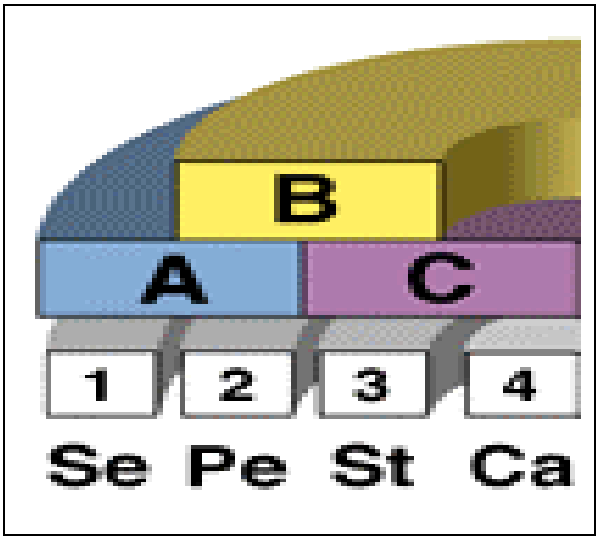

Fig. 1. The classical ABC model for the organ identity in *Arabidopsis*.
Floral organ identity is specified by the combinatorial interaction of homeotic functions A, B and C which are active in two adjacent whorls. Function A alone is expressed in whorl 1 and specifies sepals. The combined functions of A and B are expressed in whorl 2 and specifies petals; B and C are expressed in whorl 3 and specifies stamens. Function C alone is expressed in whorl 4 and specifies carpels.

1.4 MADS-box genes control floral development

Isolation and characterization of the floral homeotic genes led to the discovery that most of them belong to a conserved family of transcription factors, called the MADS-box family (Schwarz-Sommer *et al*., 1990; Yanofsky *et al*., 1990; Troebner *et al*., 1992; Coen *et al*., 1991; Mandel *et al*., 1992; Jack *et al*., 1992; Bradly *et al*., 1993; Goto *et al*., 1994; Jofuku *et al*., 1994; Theissen *et al*., 2000).

MADS is an acronym of the four founding members, MCM1, AGAMOUS, DEFICIENS, and SRF (Schwarz-Sommer *et al*., 1990). The MCM1 protein (the product of the minichromosome maintenance gene) from yeast (*Saccharomyces cerevisiae*) participates in the regulation of a- and α-cell-type specific genes and in pheromone response (Ammerer, 1990). The *AGAMOUS* (*AG*) gene controls organogenesis of stamens and carpels in *Arabidopsis*

(Yanofsky *et al*., 1990). The *DEFICIENS* (*DEF*) gene controls the formation of petals and stamens in *Antirrhinum* (Sommer *et al*., 1990). The SRF (Serum Response Factor) protein from mammals is essential for the serum-inducible transcriptional activation of the *c-fos* nuclear proto-oncogene that is involved in the transcriptional regulation of genes controlling cell growth in response to growth factors (Norman, 1988). This shows that the MADS-box transcription factors can be found not only in the plant kingdom but also in animals and yeast, where they control a variety of developmental processes. The MADS-box is a 180 bp long highly conserved region that codes for the DNA-binding domain (Pollock and Treisman, 1991).

In flowering plants, MADS-box genes constitute a large multigene family (about 80 different genes in *Arabidopsis*) whose members control diverse biological developmental processes ranging from root to flower and fruit development (Muenster *et al*., 2001). Novel MADS-box genes with regulatory roles in guard cell, trichome, root, pollen and endosperm development provided useful markers for the functional and evolutionary analyses of this gene family (Alvarez-Buylla *et al*., 2000).

Phylogeny reconstruction revealed that the MADS-box gene family is composed of several defined gene clades (Theissen *et al*., 2000; Muenster *et al*., 2001). The majority of plant MADS-box genes known so far are members of a monophyletic superclade of genes with a conserved structural organization, the so called MIKC-type, composed of a MADS (M-), an intervening (I-), a K-box (K-), and a C-terminal (C-) domain.

The MADS domain is the most highly conserved region of the proteins. In most cases, it is found at the very N-terminus of the proteins, although some MADS proteins contain additional amino acids N-terminal to the M-domain. The highly conserved M-domain of plant MADS-box proteins comprises about 60 amino acids and is the major determinant of DNA-binding. The relatively weakly conserved I-domain, directly downstream of the M-domain, comprises about

30 amino acids but is somewhat variable in length. The K-domain is defined by a conserved regular spacing of hydrophobic residues, which is proposed to allow the formation of two amphipathic helixes which are involved in selective protein dimerization. It is assumed that these amphipathic helixes interact with those of another K-domain-containing protein, thus promoting dimerization. The K-domain is not present in any of the animal or fungal MADS-box proteins known so far, indicating that the plant MADS-box proteins have a different way of interaction compared to the animal ones (Muenster *et al*., 1997). The most variable region, both in sequence and length, is the C-domain at the C-terminus of the MADS-box proteins. In a few cases the C-domain is involved in transcriptional activation, but the main function seems to be the formation of multimeric transcription factor complexes (Cho *et al*., 1999; Egea-Cortines *et al*., 1999).

1.5 The role of the B-function genes, *DEF* and *GLO*, in flower development in *Antirrhinum majus*

In *Antirrhinum* the B-function genes *DEF* and *GLO*, shown to heterodimerize via their K-domain, control petal and stamen organogenesis (Schwarz-Sommer *et al*., 1992; Troebner *et al*., 1992; Davies *et al*., 1996a; Zachgo *et al*., 1995). Upon dimerization, DEF and GLO proteins are capable of binding DNA at specific CArG-box motifs [$CC(A/T)_6GG$] in their own promoters and can boost and maintain transcription in an autoregulatory manner (Fig. 2). A *DEF* promoter mutant, the *def-chl* allele, does not show transcriptional up-regulation of the gene in petals and stamens, although the induction and basal level of expression of *DEF* and *GLO* are not affected. This promoter mutant strongly supports the hypothesis of autoregulatory maintenance of transcription.

The temporal and spatial expression patterns of *DEF* and *GLO* in a variety of mutants have indicated that, although the transcription of each gene is independently induced, the later organ specific regulation of transcription is mutually interdependent (Troebner *et al*., 1992).

Independent induction of the two genes was suggested by the following facts. First, there is a basal low level of *DEF* transcripts in flowers homozygous for the *glo-75* null allele, and a basal low level of *GLO* transcripts in flowers homozygous for the *def-gli* null allele. Second, transcripts of the two B-function genes are initially detected in young floral primordia, but there are differences in their spatial expression. In early stages of development, transcripts of *DEF* are detectable in the second and third whorl primordia. In later stages, *DEF* transcripts are found in first and fourth-whorl organs. *GLO* mRNA is found mainly in the second- and third-whorl primordial cells, but later also in fourth whorl organs (Schwarz-Sommer *et al*., 1992; Troebner *et al*., 1992; Zachgo *et al*., 1995).

The transcriptional regulation and the maintenance of *GLO* and *DEF* activity are positively correlated with the expression pattern of *DEF* and *GLO*. Because in genetically unstable lines, generating somatic revertant sectors, *GLO* transcription clearly follows the pattern of restoration of *DEF* transcription, and the *DEF* gene transcription *vice versa*. The autoregulatory model of expression of the *DEF* and *GLO* genes depends on the preexisting low level of basal expression of the GLO and DEF proteins, respectively.

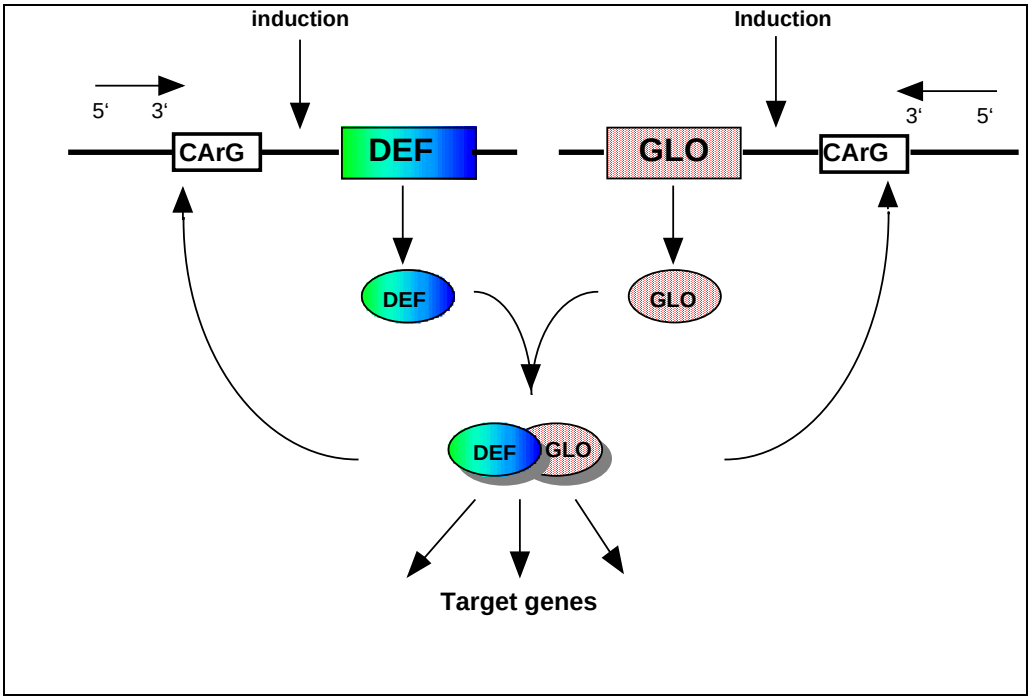

Fig.2. Autoregulatory model for DEF and GLO expression in the determination of organ identity in *Antirrhinum majus* (Troebner *et al*., 1992). The independent induction of DEF and GLO allows formation of heterodimers. The heterodimers bind to CArG motifs in the promoters of each gene and boost transcription. In addition, the heterodimers may regulate the expression of downstream target genes required for petal and stamen development.

1.5.1 Regulators for the spatial and temporal control of *DEF* and *GLO* expression

DEF and *GLO* transcripts are autoregulatory maintained at a high level only in the primordia of second and third-whorl organs, and persist in the developing petals and stamens until flower development is completed. However, the spatial and temporal expression of *DEF* and *GLO* is not uniform. Low levels of basal *DEF* transcripts are detected also in the first-whorl and fourth-whorl organs of flowers in later stages of development (see above).

The expression patterns of *DEF* and *GLO* suggests that they are independently induced and highly regulated. Indeed, several genes are known to be required for this process as deduced from genetic analysis of *Antirrhinum* mutants (Simon *et al*., 1994; Ingram *et al*., 1997;

Wilkinson *et al*., 2000).

The *FIMBRIATA* (*FIM*) gene plays a key role in the activation of the *DEF* and *GLO*, and is also needed for the activation of the C-function gene *PLE* in the center of the floral meristem. *FIM* acts in the regions between floral organs to specify the whorl patterning and maintenance of morphological boundaries (Simon *et al*., 1994; Ingram *et al*., 1997).

Mutants of two genes, *CHORIPETALA* (*CHO*) and *DESPENTEADO* (*DESP*), exhibit petaloid transformation of first whorl sepals, narrowing of vegetative organs and of all floral organs, partial female sterility, abnormal root growth and delayed germination (Wilkinson *et al*., 2000). The transformation of sepals to petaloid structure results from ectopic expression of *DEF* and *GLO* and is correlated with the ectopic expression of *FIM*.

While *FIM*, *CHO* and *DESP* are involved in the regulation of floral B- and C-function genes at the genetic level, little is known about the molecular mechanisms that control the B-function gene *DEF*.

1.5.2 RSI, a putative regulatory factor in the control of *DEF* expression

There are several mutant alleles (morphoalleles) known, which show distinct *def* mutant phenotypes. In the *def-chlorantha* allele, which is a promoter mutant, a three base pairs deletion and one base pair exchange are found 32 bp upstream of a CArG-box, which is a binding site for MADS-box transcription factors. In this mutant allele, transcription of *DEF* is strongly decreased in the second and third whorls, but the basic low levels of mRNA in sepals and carpels are not affected. This mutation indicates functional importance of this region of about 200 bp, which contains several conserved motifs for regulation of *DEF* gene expression (Schwarz-Sommer *et al*., 1992).

Using this promoter region in a yeast one-hybrid screen as bait, a putative regulator of *DEF*

was isolated, called ROSINA (RSI; M. Roccaro, personal communication).

The RSI protein is 558 amino acids long and contains several domains characteristic for transcription factors, including a b-ZIP domain at the C-terminal end and a serine rich region followed by two acidic amino acids at the N-terminus.

RSI expression is quite low compared to that of *DEF*. Expression of *RSI* is observed in primary apices and floral buds but not in leaves. In floral organs, *RSI* mRNA is first detected in stamens and at later stages in sepals and carpels.

DEF expression starts to appear in petal and stamen primordia at stage 3 of flower development and is getting localized in developing petals and stamens as judged from *in-situ* hybridization (Schwarz-Sommer *et al*., 1992). Especially in later stages of stamen development (stage 6 of *Antirrhinum* flower development), *DEF* expression is detected in incipient filaments and the connective, whereas *RSI* expression is found, complementary to *DEF*, in sporogenous tissue of the stamens (M. Roccaro, personal communication).

Taken together, the complementary expression data and the ability of RSI to bind to a region of the *DEF* promoter, suggested that *RSI* may act as a putative repressor of *DEF* activity.

1.6 Formation of multimeric complexes among MADS-box proteins

In animals and yeast, it was reported that several proteins involved in signaling, can interact by forming ternary complexes. For example, RhoA, CDC42hs, and Rac1 can activate SRF-linked signaling in this way (Hill *et al*., 1995). Another example is MCM1, a yeast MADS-box protein, which is a general regulator of cell type-specific genes in yeast and which is implicated in the response to extracellular signals. This response to pheromones is mediated by a signal transduction pathway connecting the cell surface receptors STE2 and STE3 to the

transcription factor MCM1 and STE12 (Errede *et al*., 1989; Dolan *et al*., 1989; Song *et al*., 1991). Subsequent transcriptional induction of pheromone-responsive genes is transduced through a complex consisting of STE12 and MCM1.

MADS-box proteins recognize specific DNA motifs in the control regions of other genes as homo- or heterodimers, thus regulate the transcription of these target genes. MADS-box proteins can interact with each other with a high degree of partner specificity, either as hetero- and/or as homodimers (Davies *et al*., 1996b; Fan *et al*., 1997).

But MADS proteins cannot only form homo- or heterodimers. Recently evidence for multimeric MADS protein complexes was found in *Antirrhinum* (Egea-Cortines *et al*., 1999; Egea-Cortines and Davies, 2000). The formation of a ternary protein complex between DEF/GLO heterodimer and the SQUA homodimer via their carboxy-termini, both *in vitro* and *in vivo* (in yeast) supports the observed genetic interaction between the floral meristem identity gene *SQUA* and the B-function genes *DEF* and *GLO* which is required for the establishment of the whorled phyllotaxis of floral organs in *Antirrhinum*.

Similar results were abtained in *Arabidopsis*. In addition to the A-, B- and C-function genes, the MADS-box genes *SEP1, 2,* and *3* are required to specify the organ identity of petals, stamens and carpels (Pelaz *et al*., 2000; Honma and Goto, 2001). The floral homeotic MADS-box proteins, AP1, AP3, PI and AG, form ternary and quaternary DNA-binding complexes with the SEP3 protein, in order to specify petal identity (AP3/PI/AP1/SEP), stamen identity (AP3/PI/SEP/AG) and carpel identity (AG/SEP/AG/SEP). It was the basis for the floral quartet model (Theissen, 2000; Theissen and Saedler, 2001).

A phenotype remarkably similar to that of the *sep1 sep2 sep3* triple mutant was observed from co-suppression of *FBP2* in petunia (Angenent *et al*., 1994) and antisense expression of *TM5* in tomato (Pnueli *et al*., 1994). *FBP2* and *TM5* are candidate *SEP3* orthologues from

petunia and tomato. The existence of *SEP* orthologous in distantly related eudicots, in monocots (Kang and An, 1997) and even in gymnosperms (Mouradov, 1998) suggests similar roles of the *SEP* genes in diverse species.

The *SEP 1*, *2* and *3* MADS-box genes of *Arabidopsis* were called *Im* (Identity mediating) genes (Egea-Cortines and Davies, 2000). They have counterparts in *Antirrhinum*: *DEFH72*, *DEFH200* and *DEFH84*. They are expressed in the three inner whorls of the flower and are absolutely required, together with the class A, B and C genes, to establish the organ identity of petals, stamens and carpels. Since they can interact, at the protein level, with the B-function factors AP3 and PI as well as with the C-function factor AG, they provide the link for the genetic interactions, proposed by the ABC model, in stamen and carpel specification. Thus, formation of multimeric protein complexes seems to be a common feature in the control of floral organ development.

1.7 Aims of this work

In the past developmental processes were conceived as linear hierarchical pathways, where a gene product exerted its activity on a downstream target gene of a pathway. Recently it became evident that networks of protein-protein interactions are important to specify floral organ identity (Honma and Goto, 2001; Pelaz *et al*., 2000; Egea-Cortines and Davies, 2000). The focus of the research was mainly on the interaction of the flower homeotic genes. So far, no evidence has been obtained about how the expression of the homeotic genes themselves is regulated. To get insight into the regulation of the *Antirrhinum DEF* gene, a yeast one-hybrid screen with its promoter was carried out, which yielded RSI, a putative regulator of *DEF* activity (M. Roccaro, personal communication). With the information that the multimeric

complexes of MADS-box transcription factors are a common feature for the establishment of the right organ identity, it was interesting to know whether RSI acts alone or requires other proteins to control *DEF* activity. Thus, the yeast two-hybrid system has been utilized to detect possible partners of RSI, and to understand how RSI, together with putative interacting proteins, might regulate *DEF* gene expression.

This dissertation describes the isolation and characterization of several proteins, interacting with *RSI*, in the control of *DEF* activity.

2. Materials and Methods

2.1 Materials

2.1.1 Plants

Arabidopsis thaliana	Cultivar Columbia
Antirrhinum majus	Line 165E
	Line Sippe 50

2.1.2 Bacteria strains

2.1.2.1 *Escherichia coli*

DH10B: *F⁻, mrcA,* Δ*(mrr, hsdRMS-mcrBC), Φ80dlacZ*Δ*M15,* Δ*lacX74, deoR, recA1, endA1, araD139, V(ara,leu)7697, galU, galK,* λ⁻*, rpsL, nupG*

BL21DE3: *F⁻,hsdSB(*r_B-m_B-*), gal dcm*(DE3*), ompT*

2.1.2.2 *Epicurian coli*

BL21-CodonPlus(DE3)-RIL: *E. coli* B F– *ompT hsdS*(rB– mB–) *dcm*+ Tet^r *gal* l (DE3) *endA* Hte [*argU ileY leuW* Cam^r]

2.1.2.3 *Agrobacterium tumefaciens*

GV3103 (pMP90RK): C58C1, Rif^r, Gm^r (Koncz and Schell, 1986)

2.1.3 Yeast strains

SFY526: *MATa, ura3-52, his3-200, ade2-101, lys2-801, trp1-901, leu2-3, 112, can^r, gal4-542, dal80-538, URA3::GAL1*$_{UAS}$*-Gal1*$_{UAS}$*-GAL1*$_{TATA}$*-lacZ*

HF7C: *MATa, ura3-52, his3-200, ade2-101, lys2-801, trp1-901, leu2-3, 112, gal4-542, gal80-538, LYS::GAL1*$_{UAS}$*-GAL1*$_{TATA}$*-HIS3, URA3::GAL4*$_{17\text{-}mer(\times 3)}$*-CYC1*$_{TATA}$*-lacZ*

Y187: *MATα, ura3-52, his3-200, ade 2-101, trp 1-901, leu 2-3, 112, gal4*Δ*, met⁻, gal80*Δ*, URA3GAL1*$_{UAS}$*-GAL1*$_{TATA}$*-lacZ*

Y190: *MATa, ura3-52, his3-200, ade2-101, lys2-801, trp1-901, lue2-3, 112, gal4*Δ*, gal80*Δ*, cyh^{r}2, LYS2::GAL1*$_{UAS}$*-HIS3*$_{TATA}$*-HIS3, URA3::GAL1*$_{UAS}$*-GAL1*$_{TATA}$*Z*

2.1.4 Plasmids

pBluescript II SK(+/−)	Stratagene, La Jolla/USA
pBluescript II KS(+/−)	Stratagene, La Jolla/USA
pGEX-5X-1	Pharmacia Biotech Products
pGBT9	CLONTECH®
pGAD424	CLONTECH®
pGBKT7	CLONTECH®
other vectors	in this thesis

2.1.5 Antibiotics

Name	Stock	Storage	Final concentration	Organism
Ampicilin	100 mg/ml in water	−20°C	100 μg/ml	*E.coli*
Gentamycin	25 mg/ml in water	−20°C	25 μg/ml	*A.tumefaciens*
Kanamycin	50 mg/ml in water	−20°C	50 μg/ml	*E.coli*/ *A.tumefaciens*
Rifampicin	100 mg/ml in methanol	−20°C	100 μg/ml	*E.coli*

2.1.6 Enzymes, Chemicals and Oligonucleotides

Enzymes were obtained from Roche Molecular Biochemicals (Mannheim), Biolab (England), Life Technologies (Freiburg), and used with the 10× buffer supplied.

Laboratory chemicals were obtained from following companies: Sigma (Deisenhofen), Merck (Damstadt), Roth (Karlsruhe), Serva (Heidelberg), Promega (Madison), Duchefa (Haarlem), Bio-Rad (Munich), Fluka (Neu-Ulm), Pharmacia (Freiburg) and Life Technologies. Radioisotopes were purchased from Amersham Buchler (Braunschweig).

Oligonucleotides were synthesized by Life Technologies and Metabion.

2.1.7 Primers used for cloning

Primer name	Sequence (5' – 3')	Amplification
Lac 26	TCA CAC AGG AAA CAG CTA TGA CCA TG	pBluescript®
Lac 25	CAG TCA CGA CGA TGT AAA ACG ACG G	pBluescript®
5BD	GAA TAA GTG CGA CAG CAT CAT CAT CGG	pGBT9
3BD	TGA GAA AGC AAC CTG ACC TAC AGG	pGBT9
5AD	TGG TTG GAC GGA CCA AAC TG	pGAD424
3AD	GAT GCA CAG TTG AAG TGA ACT TGC	pGAD424
EcoRV2Y16-3	CTT GAG ATA TCT CAA CTC GGC CTC CAA GC	AmGRAS
2Y16PstIEND	CCA ATG CAT TGG TTC TGC AGT CAC CAT CAA ACA CAT ACT CA	AmGRAS
EL1	TTG CGT ACT GAC GGA TTC ATC GTT G	EMBL3
ER2	ATA TAT GCT TTC CAT TCC ATG GGG A	EMBL3
BGAI	GGA CTC GAG CCA GGA GGA GAG ATC	AmGRAS
Xba2Y16-3	GCT CTA GAA CTC GGC CTC GGC CTC CAA GCC GAT GT	AmGRAS
BamHI2Y16-5	ACG GGA TCC ATG AAA AGG GAT AGC AGT	AmGRAS
PstBGA5	CCA ATG CAT TGG TTC TGC AGT CAG GGT GGG GGT TGA GGG TA	AmGRAS
2RS	GGG GAA GAG TTG ATG ATT AAT	Promoter
5RS	CAA GAA TAT CTC ATC AAA AGA	Promoter
6RS	ATG TAT AAG TTA TTG GCA TTA	Promoter
5RS-1	GGT TCA CTG TCC GGT TCG GTT	Promoter
6RS-1	ATT GGC ATT AGA GAC CAA GTT	Promoter
EB	CCG GGA GCT GCA TGT GTC AGA GG	pGEX5X-1
EF	GGG CTG GCA AGC CAC GTT TGG TG	pGEX5X-1
Y6Xho-3	CCG CTC GAG ATC AGA ATT GGG AAA AGA	Y6
2Y16L-1	GGG GTA CCT CAA CTC GGC CTC CAA GC	AmGRAS
2Y16L-4	TTG CTG CAT CGG CTT GGA GGC CGA GTT	AmGRAS
2Y19Xho-3	CCG CTC GAG GTT GCT CTT TCT CTT TGT	2Y19
2Y14-3SmaI	TCC CCC GGG ATT TTC ATC CAA AAG	2Y14
2Y6-3SmaI	TCC CC GGG GCA TTT ATG TTG AAC	2Y6
Y13-3SmaI	TCC CCC GGG GTT GTT GTA CGA ATG	Y13
35S	GAC GCA CAA TCC CAC TAT CCT TCG	pGPTV-BAR
pAnos	TAT TAC ATG CTT AAC GTA ATT CAA CAG	pGPTV-BAR

2.2 Methods

2.2.1 Isolation and purification of nucleic acids

2.2.1.1 Small scale plasmid DNA isolation from *E.coli* by the alkaline lysis method

For small scale plasmid DNA isolation and purification, a single bacterial colony was transferred to 3-5 ml of LB medium and incubated overnight at 37°C with vigorous shaking. Overnight cultures were poured into 1.5 ml eppendorf tube and centrifuged down at 2,000 g for 5 min (5,000 rpm in a Heraeus #3325 rotor). The supernatant was removed by aspiration,

and the bacterial pellet was left as dry as possible. The bacterial pellet was resuspended in 250 µl of ice-cold alkaline lysis solution I by vigorous shaking. The same volume of lysis solution II was added and gently inverted five times. All the complexes including bacterial proteins, broken cell walls, and denatured chromosomal DNA were efficiently precipitated with solution III containing potassium ions. After the debris has been removed by centrifugation at maximum speed, plasmid DNA was recovered from the supernatant using a QIAprep column. The supernatant, after addition of solution III, was decanted into QIAprep column and centrifuged for 1 min at high speed (12,000 rpm in a Heraeus #3325 rotor). The flow-through was discarded, the column washed with 0.75 ml of ethanol containing PE buffer (provided from QIAGEN®) and the residual wash buffer was removed by centrifugation at maximum speed (12,000 rpm in a Heraeus #3325 rotor). The plasmid DNA was eluted from the column by adding 50 µl of elution buffer (EB) and centrifugation.

LB medium/l: 10 g of bacto tryptone, 5 g of bacto yeast extract, 10 g of NaCl, and 15 g of bacto agar (for plates only)
Solution I: 50 mM Tris-HCl pH 8.0, 10 mM EDTA , 100 µg of RNase A/ml
Solution II: 0.2N NaOH, 1% SDS
Solution III: 3M KOAc pH. 5.5
Elution buffer (EB): 10 mM Tris-HCl, pH 8.0.

2.2.1.2 Large scale plasmid DNA isolation from *E.coli* by the alkaline lysis method

The principle of a large scale plasmid DNA isolation is similar to that of the small scale plasmid DNA isolation. Volumes of solutions were accordingly scaled up and a different column (NUCLEOBOND®) was used. Thirty to fifty milliliter of LB overnight culture obtained from a single colony was centrifuged down at 4,000 g (5,000 rpm in a Heraeus christ Typ-Nr. 4400 roter). The bacterial cells were resuspended in 4 ml of buffer S1 for 5 minutes. The same volume of S2 buffer was added, and the suspension was immediately mixed by inverting the tube 5 times. This mixture was incubated for 5 minutes at RT. Four milliliters of the S3 solution was then added and the mixture was incubated for 5 min on ice to precipitate chromosomal DNA. The bacterial lysate was clarified by centrifugation at maximum speed for 30 minute and by filtration using miracloth. With centrifugation, SDS was also removed from the buffer S3 solution (white precipitate). The NUCLEOBOND® column was equilibrated with 2 ml of buffer N2. The cleared bacterial lysate was poured onto an equilibrated column and the flow-through was discarded. The NUCLEOBOND® column was washed twice with 4

ml of N3 buffer and the plasmid DNA was eluted by adding 4 ml of the N5 buffer. The purified plasmid DNA was precipitated with 0.8 volume of isopropanol, and centrifugation for 30 min at high speed (10,000 rpm in a Beckman JS-13.1 rotor, 19,100 g) at 4°C. The DNA pellet was dried for 5 min at RT and resuspended in an appropriate volume of elution buffer (ca. 500 µl -1 ml).

Buffer S1: 50 mM Tris-HCl (pH8.0), 10 mM EDTA, 100 µg RNase A / ml
Buffer S2: 0.2 M NaOH, 1% SDS
Buffer S3: 2.8 M KOAc, pH 5.1
Buffer N2 : 100 mM Tris, 15% EtOH, 900 mM KCl, pH 6.3
Buffer N3 : 100 mM Tris, 15% EtOH, 1150 mM KCl, pH 6.3
Buffer N5: 100 mM Tris, 15% EtOH, 1000 mM KCl, pH 8.5.

2.2.1.3 Large scale genomic DNA isolation from *Antirrhinum majus*

Three grams of plant material was frozen in liquid nitrogen and ground using mortor and pestle. The ground power was thawed in an extraction buffer (3×SSC, 0.1 M EDTA, 0.1 M Na-diethyldithiocarbonate, and 10% SDS) as described with some modification (Coen *et al*., 1986). The preparation was extracted once with chloroform, once with phenol:chloroform (1:1), and again with chloroform. Nucleic acids were precipitated with 0.6 volume of isopropanol, redissolved in 5 ml of TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA) and treated with RNase A (25 µg/ml) for 30 min. 5 M NaCl was added to the solution, followed by an equal volume of 2% cetyltrimethylammoniumbromide (CTAB), 50 mM Tris-HCl (pH 8.0), 10 mM EDTA. The precipitate was washed with 70% EtOH and air-dried for 1 hour. The pellet was resuspended in 200 µl of TE buffer.

2.2.1.4 Small scale isolation of genomic DNA for PCR analysis

Plant leaf discs were collected in sterile eppendorf tubes containing 300 µl of extraction buffer, incubated for 30 to 60 min at RT. The leaf discs were ground roughly using a plastic macerator and mixed well by vortexing. The same volume of phenol:chloroform(1:1, pH 8.0) was added and incubated for 15 min at RT. The resulting suspension was centrifuged down at 13,000 g (14,000 rpm in a HERAEUS #3754 rotor) for 10 min. The supernatant was transferred into a new eppendorf tube and precipitated with equal volume of isopropanol. Precipitated genomic DNA was centrifuged down at 13,000 g, washed with ice-cold 70% EtOH and air-

dried for 10 min. The pellet was redissolved in 200 µl of TE and 2 µl was taken for the PCR reaction (Edwards *et al*., 1991).

Extraction buffer: 200 mM Tris-HCl (pH7.5), 250 mM NaCl, 25 mM EDTA, 0.5% SDS
TE: 10 mM Tris-HCl(pH 8.0), 1 mM EDTA.

2.2.1.5 Isolation of total RNA from plant tissue

To isolate intact RNA from plant tissues, guanidine thiocyanate was used to disrupt cells, solubilize their components, and to denature endogenous Ribonucleases (RNases) simultaneously. RNases are very stable and active enzyme that generally do not require cofactors to function. To minimise the ribonuclease activity, sterile plastic ware and sterilized glassware (baking for 8hrs at 180°C) was used.

One gram of freshly-collected plant tissues was ground in a mortar. The tissue powder was suspended in 9 ml of buffer R1 containing 9 µl of buffer R2 and homogenized 3-5 times with a POLYTRON® for 15 sec. All operations were done quickly and keeping cold. The homogenate was mixed by adding of 720 µl of buffer R3, and incubated on ice for 15 min. Thereafter, 9 ml of ice-cold buffer R4 was added, and the sample was incubated on ice for additional 15 min. The homogenized cells were collected by centrifugation at 15,000g for 30 min at 4°C. The supernatant was decanted on ice into a sterile 50 ml tube and precipitated by adding 0.8 volume of isopropanol for 5 min. The RNA pellet was collected in a COREX® tube by centrifugation at 10,000 rpm in a Beckman JS-13.1 rotor. The pellet was dissolved in 16 ml of ice-cold buffer R5. To minimize column clogging, a pipet with a cut-off tip was used for a thorough resuspension of the pellet and an additional incubation at 60°C was applied for 5–10 min. If the suspension still contained particulate matter, it was recentrifuged prior to mixing with buffer R6 and loading onto a column. Completely dissolved RNA solution was mixed with 4 ml of buffer R6, applied onto QIAGEN-tip 500 column (preequilibrated with 10 ml of buffer QAT) and allowed to enter the resin by gravity flow. The QIAGEN-tip 500 column was washed with 30 ml of buffer QA. The RNA was eluted with 20 ml of buffer QRU. Eluted RNA was precipitated with 1 volume of isopropanol for 10 min on ice and collected by centrifugation at 10,000 rpm at 4°C. The pellet was washed with 80% EtOH, air dried for 10 min at RT and dissolved in DEPC-treated H_2O.

Buffer R1: 4 M Guanidine thiocyanate(GIT), 100 mM Tris-HCl, 25 mM MgCl, 25 mM EDTA, pH7.5

Buffer R2: β-mercaptoethanol

Buffer R3: 25% Triton X-100

Buffer R4: 3 M NaAc, pH 6.0

Buffer R5: 20 mM Tris-HCl, 1 mM EDTA, pH 8.0

Buffer R6: 2 M NaCl, 250 mM MOPS, pH 7.0

Buffer QAT: 400 mM NaCl, 50 mM MOPS, 15% ethanol, 0.15% Triton X-100, pH 7.0

Buffer QA: 400 mM NaCl, 50 mM MOPS, 15% ethanol, pH 7.0

Buffer QR: 1.2 M NaCl, 67 mM MOPS, 20% ethanol, pH 6.7

Buffer QRU: 900 mM NaCl, 50 mM MOPS, 15% ethanol, 6 M urea (added to Buffer QR just prior to use), pH 7.0.

2.2.1.6 Purification of mRNA from total RNA using DYNABEADS® OLIGO(dT)$_{25}$

Dynabeads® Oligo (dT)$_{25}$ kit (Deutsche Dynal GmbH) is designed for the rapid isolation of pure, intact poly (A)$^+$ RNA. Dynabeads are uniform, superparamagnetic polystyrene beads, 2.8 µm in diameter. Chains of deoxythymidylate, 25 nucleotides long, have been covalently attached to the surface of the beads.

This method of mRNA purification is based on base pairing between the poly (A) residues of mRNA and oligo (dT) residues covalently coupled to the surface of the beads. The suggested protocol uses 75 µg total RNA as starting material. The approximate capacity of the beads is 2 µg per mg, therefore 1 mg beads could purify sufficient amount of mRNA for any application.

Up to 75 µg of total RNA was adjusted to 100 µl with DEPC treated water. Less concentrated RNA solution (more dilute than 75 µg/100 µl) were simply mixed with an equal volume of 2× binding buffer. RNA mixture was heated at 65°C for 2 minutes to disrupt secondary structures. One milligram of Dynabeads was taken into MPC®-E-1 (magnetic particle concentrator) and washed with 2× binding buffer. Total RNA was added to the bead suspension, mixed gently, left to hybridize for 3-5 minutes, and beads were separated from supernatant using MPC®-E-1 magnet. The beads were washed twice with 200 µl washing buffer. Pure mRNA was eluted by adding 100 µl of elution buffer, followed by heating at 65°C for 2 minute and separating immediately using MPC®-E-1. The eluted mRNA was used immediately or stored frozen at -70°C until needed.

2× Binding buffer: 20 mM Tris-HCl, 2 mM EDTA, 1 M LiCl, pH 7.5

1× Washing buffer: 10 mM Tris-HCl, 1 mM EDTA, 0.15 M LiCl, pH 7.5
Elution buffer: 2 mM EDTA, pH 7.5.

2.2.1.7 Spectrophotometric quantification of DNA and RNA

For quantitating DNA or RNA, reading was taken at wavelength of 260 nm and 280 nm. The reading at 260 nm allows calculation of the concentration of nucleic acids in the sample. OD of 1 corresponds to approximately 50 µg/ml for double-stranded DNA, 40 µg/ml for single-stranded DNA and RNA. The OD_{260}/OD_{280} value provides an estimate of the purity of the nucleic acid. Pure DNA or RNA have OD_{260}/OD_{280} values of 1.8 and 2.0, respectively. If there is a contamination with protein or phenol, the OD_{260}/OD_{280} would be significantly less than the values given above.

2.2.1.8 Separation of DNA by agarose gel electrophoresis

Electrophoresis through agarose gels is a standard method used to separate and purify DNA fragments. The agarose concentraion depends on the size of the fragments to be resolved. The gel was solidified with 1× TBE buffer containing 5 µl of ethidium bromide (5 mg/ml). DNA fragments were mixed with 1/10 of 10× Gel loading buffer and separated on the agarose gel. Electrophoresis was performed at 5V/cm using 1× TBE buffer. Separated DNA fragments were visualized on a transilluminator by fluorescence under UV light (254 nm). DNA size marker was loaded to estimate the size of fragments.

10× Gel loading buffer: 50% glycerol, 1 mM EDTA, 0.25% bromophenol blue, 0.25% xylene cyanol.
1× TBE buffer: 90 mM Tris-HCl (pH 8.3), 90 mM boric acid, 2.5 mM EDTA.

2.2.1.9 Separation of RNA through formaldehyde gels

For the electrophoretic separation of RNA fragments, a 0.8% formaldehyde gel was prepared. To prepare 100 ml of formaldehyde agarose gel, 0.8 g of agarose was dissolved in 10 ml of 10× MOPS buffer and 73 ml of DEPC-treated water. Once the gel was cooled down to ca. 60°C, 17 ml of formaldehyde was added in a fume hood with stirring. The gel was prepared for horizontal electrophoresis. Poly $(A)^+$ RNA sample (2 µg/2 µl) was mixed with 15

µl of a sample buffer and 2 µl of a loading buffer, then denatured by heating at 60°C for 10 min. The gel was run at ca 2V/cm in 1× MOPS buffer until BPB marker has migrated ca. 6 cm (approximately 5 hrs) in fume hood.

Sample buffer: 700 µl of deionized formamide, 270 µl of 35% formaldehyde, 30 µl of 10× MOPS.

Deionization of formamide: For suspension deionization of formamide, 0.5% of Serdolit®MB-1(SERVA # 45500) resin was added. The suspension was slowly agitated for 1 hr at room temperature and filtered. After filtration, the purified formamide was stored at -20°C.

2.2.1.10 Transfer of nucleic acids onto nylon membranes

Nucleic acids were transferred onto nylon membrane (Hybond$^+$, Amersham) by capillary blotting. DNA was separated on a 0.8% agarose gel in fresh 1× TBE buffer and transferred to a nylon membrane using 20× SSC. The RNA was separated under denaturing conditions by agarose gel eletrophoresis. To remove the formaldehyde, the agarose gel was washed with distilled water several times and transferred to a nylon membrane using 20× SSPE. The membrane with transferred nucleic acid was washed with 6× SSC, air-dried and exposed to UV light (Stratalinker, Stratagene) to cross-link nucleic acids.

20× SSC: 3 M NaCl, 0.3 M sodium citrate, pH 7.0.
20× SSPE: 3.6 M NaCl, 20 mM EDTA, 200 mM NaH_2PO_4, pH 7.4.

2.2.1.11 Radiolabeling of DNA probes with [α-^{32}P] dCTP

The klenow fragment of *E.coli* DNA polymerase I lacks 5'→ 3' exonuclease activity, so the radiolabeled product is synthesized exclusively by primer extension. A DNA fragment to be labeled was diluted with distilled H_2O to a concentration of 50 ng in 21 µl and denatured by boiling for 5 min. The denatured DNA was mixed with 4.2 µl of an oligonucleotide mixture (dATP, dTTP, and dGTP) 1.5 µl of BSA(1mg/ml), 3 µl of [α-^{32}P] dCTP and 1U of Klenow fragment. The mixture was incubated at 37°C for 1-2hrs. Labeled DNA fragment was purified using the QIAquick nucleotide removal kit according to the provided protocol.

2.2.1.12 Southern blot hybridization

Genomic DNA (3-4 μg) was digested with different restriction enzymes, and the resulting fragments were separated according to size by electrophoresis through an agarose gel. To improve transfer efficiency, DNA was depurinated by soaking the gel for 10 min in several volumes of 0.25 N HCl, then denatured in denaturation solution for 30 min. In each step, the agarose gel was rinsed briefly with deionized water. The DNA was then soaked with neutralization solution for 30 min and transferred onto nylon membrane by capillary blotting. The membrane was briefly washed with 6 × SSC, air dried, baked for 30 min at 80 °C and used for hybridization. The Southern blot hybridization (Southern, 1975) was performed at 68 °C. Before the hybridization, the nylon filter was prehybridized for 30 min. During the prehybridization, ^{32}P-labeled probe and 50μl/ml of salmon sperm DNA was denatured and added to the hybridization solution. After pouring off the prehybridization solution, the filter was incubated in hybridization solution overnight and washed with a series of washing buffers. The efficiency of washing was monitored with a Geiger counter. The filter was covered with transparent plastic foil and exposed to a X-ray film. The film was normally developed after 3-5 days of exposure at -70 °C with an intensifying screen.

Denaturation solution: 0.5 N NaOH, 1.5 M NaCl
Neutralization solution: 3 M NaCl, 0.5 M Tris-HCl, pH 7.5
Prehybridization solution: 6× SSPE, 3% SDS, 0.02% PVP, 0.02% Ficoll 400
Hybridization solution: 3× SSPE, 1% SDS, 0.02% PVP, 0.02% Ficoll 400
Salmon Sperm DNA: Salmon Sperm DNA (Sigma type III sodium salt) was dissolved in TE, pH7.6 at a concentration of 5 mg/ml. If necessary, the solution was boiled in a microwave oven for 5-10 min, then stirred for 3- 4 hrs to dissolve the DNA
Washing solution: 2× SSPE, 0.1% SDS or 1× SSPE, 0.1% SDS or 0.1× SSPE, 0.1% SDS.

2.2.1.13 Northern blot hybridization

Northern blot hybridization was carried out with RNA immobilized on a nylon filter and a radiolabeled probe at 42 °C. All procedures were the same as in the Southern blot hybridization protocol, but the solution was as follows.

Pre/hybridization solution: 50% deionized formamide, 5× SSPE, 10× Denhardt mix, 0.5% SDS, 100 μg/ml of salmon sperm DNA

Non-stringent washing solution: 2× SSPE, 0.1% SDS at 25°C

Stringent washing solution: 1× SSPE, 0.1% SDS at 40°C.

2.2.2 Enzymatic modifications of DNA

2.2.2.1 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 1-2hrs with 10 units of enzyme at an appropriate temperature (mostly at 37°C), and stopped by heat inactivation at 75°C for 10 min.

2.2.2.2 Ligation of DNA fragments

Ligation of DNA fragments to a linearlized 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 alkaline phosphatase. 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.

2.2.3 Bacterial Transformation

2.2.3.1 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.

2.2.3.2 Elcetroporation of bacterial cells

Frozen elecrocompetent 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 1.5 hrs, 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 $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose (filter sterilized, added whenever it is needed).

2.2.4 Methods for proteins analysis

2.2.4.1 Purification of GST-fusion proteins from *E.coli*

Fusion proteins expressed from pGEX vectors (Promega) contain a glutathione S-transferase (GST) moiety and can be purified by affinity chromatography on glutathione agarose (G4510, SIGMA) (Smith and Johnson, 1998). GSTs are a class of enzymes that utilize glutathione (γ-glutamylcysteinylglycine) as a substrate to inactivate toxic small molecules via formation of mercapturic acids. Because the affinity of GST for its substrate is in the submillimolar range, immobilization of glutathione on an agarose matrix makes a highly efficient affinity chromatography resin.

cDNA fragments from pGAD424 and pACT2 plasmids were suhcloned into pGEX-5X-1, transformed into *Escherichia coli* DH10B for propagation and retransformed into *Escherichia coli* BL21 or *Epicurian coli* BL21-CodonPlus (DE3)-RIL for purification of glutathione S-transferase (GST)-fusion protein.

The 25 ml overnight culture containing 100 µg/ml ampicillin was used to inoculate a 500 ml culture, and grown at 30°C until the OD_{600} is 0.8-0.9 (ca. 2-2.5 hr). A 1 ml sample was immediately taken before induction (noninduced cells), centrifuged down and resuspended in 2× SDS gel loading buffer. The sample was frozen at -20°C until needed for SDS-PAGE. The rest of the cells was induced by adding IPTG to a final concentration of 0.5 mM. The culture was grown for an additional 2.5-3hrs. A second 1 ml sample was taken and stored at -20°C as done above. The induced cells were harvested by centrifugation at 4000 g for 15 min, resuspended in 9 ml of MTBS buffer containing 1% Triton, 1 mM PMSF, 1 mg/ml lysozyme and incubated on ice for 15 min. To lyse cells, sonification (Branson sonifier model B-12/B-15)

was used for 3×15 seconds continuously at 3-4 power. The lysed cells were centrifuged down at higher speed (10,000 rpm in a Beckman JS-13.1 rotor). The supernatant was combined with 1 ml of swolen glutathione-agarose (7 mg glutathione agarose in 5 ml MTBS buffer was rotated gently at 4°C O/N) for 30 min at 4°C. Bound proteins were precipitated at 1,000 rpm in a Heraeus CHRIST Typ-Nr. 4400 roter. For analysis, a third 1 ml sample was taken from the supernatant. The bound proteins were in the pellet together with agarose beads. To get rid of unbound proteins, beads were extensively washed 5 times with 2 ml of MTBS buffer at 800 rpm in a Heraeus CHRIST Typ-Nr. 4400 roter. Specifically bound proteins were eluted with 100 µl of elution buffer after 1-2 hr incubation, and eluted again after overnight incubation at 4°C. The amount of protein was estimated by SDS-polyacrylamide electrophoresis with a defined amount of BSA, and the protein was stored at -70°C until needed.

MTBS buffer/l: 8.77 g NaCl, 2.85 g Na_2HPO_4, 0.55 g NaH_2PO_4, pH 7.3.
Elution buffer: 10 mM reduced glutathione(SIGMA), 50 mM Tris-HCl, pH 8.0.

2.2.4.2 SDS-polyacrylamide gel electrophoresis of proteins

The analytical electrophoresis of proteins was carried out in SDS-polyacrylamide gel that ensured denaturation of the proteins and minimized aggregation. The denatured polypeptides bind SDS and become negatively charged, because the amount of bound SDS is almost proportional to the size of the polypeptide and is mearly independent of its sequences.

The electrophoresis was carried out with a discontinuous buffer system in which the stacking gel contains Tris-HCl (pH 6.8), the upper and lower reservoirs contain Tris-glycine (pH 8.3), and the resolving gel contains Tris-HCl (pH 8.8) to increase the resolution of SDS-polyacrylamide gels.

A minigel apparatus (Hoefer), which contains 10x8 cm glass plates was routinely used for protein analysis and *in vitro* GST pull-down assay. Once the resolving gel has been poured, the isobutanol was used to overlay the solution. This overlay prevents oxygen from diffusing into the gel and inhibiting polymerization. After polymerization was completed (ca. 30 min), the overlay was poured off and the stacking gel was poured up to the top of the glass plates. Immediately, a clean comb (1.6 cm) was inserted and the gel was polymerized in a vertical position.

Samples were mixed with a loading buffer, boiled for 5 min to denature proteins and loaded onto the gel. The electrophoresis was carried out at a constant current of 15 mA when the dye was in the stacking gel and 30 mA when the dye reached to the separating gel.

Resolving gel (10%)/30 ml	dH_2O	11.88 ml
	30% acrylamide mix	10.02 ml
	1.5 M Tris-HCl (pH8.8)	7.50 ml
	10% SDS	0.30 ml
	10% APS	0.30 ml
	TEMED	20.00 µl
Stacking gel (5%)/10 ml	dH_2O	6.80 ml
	30% acrylamide mix	1.70 ml
	1 M Tris-HCl (pH 6.8)	1.25 ml
	10% SDS	100 µl
	10% APS	100 µl
	TEMED	6 µl
2x SDS loading buffer:	100 mM Tris-HCl(pH 6.8), 200 mM DTT, 4% SDS 0.2% bromophenol blue, 10% glycerol	
Running buffer:	3% Tris, 14.4% Glycine, 1% SDS.	

2.2.4.3 Staining of gels with coomassie brilliant blue

Coomassie brilliant blue is an aminotriarylmethane dye that forms strong but not covalent complexes with proteins by a combination of van der Waals forces and electrostatic interactions with NH_3^+ groups. After electrophoresis in a SDS-polyacrylamide gel, 1% coomassie brilliant blue was used to stain proteins for 30 min at RT. The stained gel was washed several times with a destaining solution.

Coomassie staining solution: 1% Coomassie brilliant blue R-250, 45% methanol, 10% acetic acid.

Destaining solution: 45% methanol, 10% acetic acid.

2.2.4.4 Electroblotting of proteins

Electrophoretic transfer of proteins was carried out perpendicularly from the direction of travel of proteins through the separating gel. The gel and positively charged nylon membranes (Hybond N^+, Amersham) were sandwiched between pieces of a Whatman 3MM paper that have been soaked in a transfer buffer. The sandwich was then placed between graphite plate electrodes, with nylon membrane on the anodic side. Transfer of proteins from the gel to membrane was carried for 1.5 hr to 2 hr. The gel was dried for 1hr at RT, and the membrane was then used for Western blot with antibody.

10× Western transfer buffer: 1.5 M Glycine, 0.2 M Tris base.

2.2.4.5 *In vitro* translation and transcription

For *in vitro* translation and transcription, TNT® Coupled Reticulocyte Lysate Systems (Promega);offering an eukaryotic *in vitro* coupled trancription/translation system, was used. The circular plasmid DNA was phenol:chloroform extracted and ethanol precipitated before use in a transcription/translation reaction. The following is an example of standard reactions using [^{35}S] methionine.

TNT® Rabbit Reticulocyte Lysate		25 µl
TNT® Reaction buffer		2 µl
TNT® RNA T7 polymerase		1 µl
Amino acid mixture, minus methionine, 1mM		1 µl
[^{35}S] methionine (37TBq/mmol at 10mCi/ml)		2 µl
RNAse inhibitor		1 µl
DNA		1 µg
ddH_2O	to a final volume of	50 µl

2.2.4.6 *In vitro* GST fusion protein pull-down assay

cDNA fragments cloned in pGAD424 and pACT2 were subcloned in pGEX-5X-1 and used for purification of glutathione S-transferase(GST) fusion proteins. A cDNA fragment of RSI (obtained from Dr. M. Roccaro) served as a template to synthesize [^{35}S] methionine-labeled proteins in TNT® Coupled Reticulocyte Lysate Systems (Promega). GST fusion proteins (10 µg,

estimated using BSA protein staining), a control GST protein and the empty matrix (glutatione-agarose beads alone) was immobilized with glutathione-agarose in 150 µl of a binding buffer (100 mM NaCl, 20 mM Tris· HCl pH. 7.5, and 0.1% Nonidet P 40) for 30 min at 4°C. The beads were centrifuged down at 2,000 g for 1 min and washed with 500 µl of the binding buffer. In order to reduce unspecific binding, the beads were incubated using 300 µl of a blocking buffer (5 mg/ml of a milk powder) for 30 min at 4°C , then washed again with 500 µl of the binding buffer. Equal amounts (3 µl) of the ^{35}S-labeled RSI protein were incubated with glutatione-agarose coupled to GST fusion proteins, the control GST protein and empty matrix in 200µl of the blocking buffer (1 mg/ml) for 1 hr at 4°C. After removal of the supernatant, the beads were extensively (3 times) washed with binding buffer, and together with the supernatant fractions, separated on a 10% SDS-polyacrylamide gel. The ^{35}S-labeled RSI protein was detected by autoradiography.

2.2.5 Methods for yeast two-hybrid system

2.2.5.1 Yeast two-hybrid screen

The vectors and strains provided in the MATCHMAKER Two Hybrid System (CLONTECH ®) were used for screening of the *Antirrhinum* cDNA expression Library obtained from Dr. H. Sommer. A 1 l culture of a yeast strain Y190 in YPD medium, containing the appropriate bait, was transformed with a total of 100 µg of cDNA (1:1 of random-primed cDNA in pACT2 and oligo(dT)-labeled cDNA in pGAD424) library, and 900 µg of carrier DNA (Schiestl *et al*., 1989) by the lithium acetate method (Gietz *et al*., 1992). After additional growth in 1 l of YPDA medium, the cells were pelleted, resuspended in 10 ml of YSD medium without glucose and plated onto six 24 cm square plates containing a medium to select for all possible interactors (Trp^-Leu^-). Transformation efficiency was also determined by growing a small aliquot on medium lacking Trp and Leu. Colonies from 6 plates scraped off after growing for 2 days. They were plated again onto fifty-five 15 cm square plates containing 3-AT (25-30 mM) to select for histidine prototrophy ($Trp^-His^-Leu^-$). Approximately 100,000 cells per plate were plated, grown for 10-14 days, picked and re-streaked on selective medium for conformation. β-Galactosidase activity was assayed on filters as described (Breedon and Nasmyth, 1985).

The yeast strains used in this study have been described previously:

Y190 (Harper *et al*., 1993), HF7c (Feilotter *et al*., 1994) and SFY526 (Bartel *et al*., 1993).

YPD media/l: 20 g of Difco peptone, 10 g of yeast extract, 18 g of agar (for plates only).
Add H_2O to 950 ml. Adjust pH to 5.8, autoclave, and cool to ~55 °C .
Add dextrose (glucose) to 2% (50 ml of a sterile 40% stock solution).

YPDA medium: To 1 liter of YPD medium, add 15 ml of filter-sterilized 0.2% adenine hemisulfate to a final concentration of 0.003%.

2.2.5.2 Plasmid isolation from yeast

Shuttle plasmids which replicate in both *Escherichia coli* and *Saccharomyces cerevisiae* can be isolated from yeast, and used to transform *E.coli*. Plasmid DNA isolated from yeast (Hoffman *et al*., 1987) is often contaminated by yeast genomic DNA, therefore retransformation into *E.coli* is necessary.

A single colony, grown on a selective YSD media lacking Trp and Leu for 3 to 4 days, was inoculated into 5 ml of YPD liquid medium and grown overnight at 30 °C. Freshly grown yeast cells were centrifuged down at 2,000 g for 5 min. The pellet was resuspended with 0.2 ml of a yeast lysis solution, an equal volume of phenol/chloroform (1:1 pH 8.0) and 0.3 g of acid washed glass beads (Sigma, G-8772) were added, and mixed vigorously by vortexing. The suspension was centrifuged down at 13,000 g for 15 min at RT. The supernatant was precipitated with 0.8 volume of isopropanol. The pellet was washed with 70% EtOH, air-dried and dissolved in 200 µl of TE.

Yeast lysis buffer: 2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA

YSD media/l: 6.7 g of Difco yeast nitrogen base, 20 g agar (for plates only)
Add H_2O to 850 ml. Add 100 ml of the appropriate sterile 10× dropout solution.
Adjust pH to 5.8, autoclave, and cool to ~55 °C. Then add dextrose (glucose)
to 2% (50ml of a sterile 40% stock solution)

10× Dropout solution/l: 300 mg of L-Isoleucine, 1500 mg of L-Valine, 200 mg of L-Adenine hemisulfate salt, 200 mg of L-Arginine HCL, 200 mg of L-Histidine HCL monohydrate, 1000

mg of L-Leucine, 300 mg of L-Lysine-HCL, 200 mg of L-Methionine, 500 mg of L-Phenylalanine, 2000 mg of L-Threonine, 200 mg of L-Tryptophan, 300 mg of L-Tyrosine, 200 mg of L-Uracil.
10× Dropout solution was autoclaved and stored at RT.

2.2.5.3 β-galatosidase filter assay

As a result of a two-hybrid protein-protein interaction, the GAL4 transcriptional activator is functionally reconstituted and activates transcription of reporter genes (*LacZ* and *HIS3*) carrying upstream a GAL 4 binding site. Screening for expression of the *LacZ* reporter gene, which has a different promoter as the *HIS3* reporter gene, eliminates many of the false positives that arise in a yeast two-hybrid screening (Breedon and Nasmyth, 1985).

A single yeast colony was streaked and grown on a filter and placed on YSD media with an appropriate selection for 2 to 3 days at 30°C. The filter (nylon membrane) was permeabilized with liquid nitrogen for 10 sec. The filter was carefully placed, colony side up, on a filter prewetted with β-galactosidase filter assay buffer. The appearance of blue color was checked periodically during incubation at 30°C.

β-galactosidase filter assay buffer: 10 ml of Z-buffer, 27 µl of β-mercaptoethanol, 167 µl of X-gal (20 mg/ml in DMF)

Z-buffer/l: 16.1 g of $Na_2HPO_4 \cdot 7H_2O$, 5.50 g of $NaH_2PO_4 \cdot H_2O$, 0.75 g of KCl, 0.246 g of $MgSO_4 \cdot 7H_2O$, pH 7.0.

2.2.5.4 β-galatosidase liquid assay

A single yeast colony was transferred into 5 ml of a selective YSD liquid media (Trp^-Leu^-), and incubated overnight at 30°C with shaking (250 rpm). From the overnight culture, 2 ml was diluted with 8 ml of YPD liquid media and grown for ca. 3 hrs until the OD_{600} reached 0.5-0.8. After vortexing to disperse cell clumps, the exact OD_{600} was recorded. One and half milliliter of culture was centrifuged and washed carefully with Z buffer. The pellet was resuspended in 300 µl of Z buffer, thereby concentrating the cells 5- fold. 100 µl of the cell suspension were transferred into a new tube, liquid nitrogen permeabilized for 1 min, and thawed in a 37°C water bath for 1 min. As a control, a tube with 100 µl of Z buffer was used. For each tube, 0.7

ml of Z buffer plus β-mercaptoethanol was added, and immediately 0.16 ml of ONPG in Z buffer (4 mg/ml) was added. After the yellow color developed, 0.4 ml of 1 M Na_2CO_3 was added to the tubes. The elapsed time in minutes was recorded. Cell debris was pelleted by centrifugation, and the supernatants were carefully transferred into clean cuvettes. The units of β-galactosidase enzymatic activity was calculated using the following formula

$(1000 \times A_{420})/T \times V \times A_{600}$

A_{420}: absorbacce of yellow colour developed by the reaction at 420 nm

A_{600}: the turbidity or absorbance of the yeast culture at 600 nm

T: time of the reaction in minutes

V: 0.1 ml×concentration factor (0.1 ml was used for the reaction).

2.2.5.5 Small scale yeast co-transformation

A single yeast colony grown in YPD medium was transferred into 10 ml of the YPD liquid media, and grown overnight. Freshly grown yeast cells were collected by centrifugation at 2000 g for 15 min, washed once with 1/10 volume of distilled water and once with 1/10 volume of 1×LiAc/TE solution. Plasmid DNA together with 5 µl of DMSO and 5 µl of carrier DNA (10 mg/ml) was mixed well, thereafter 50 µl of yeast cells and 350 µl of 1× PEG/LiAc/TE were added. The mixture was incubated at 30°C for 30 min and heat-shocked at 42°C for 15 min. The cells were centrifuged down and resuspended with 500 µl of distilled water. From the resuspended cells, 50 – 100 µl were spread onto selectable YSD plates and co-transformed cells were visible after approximately 4-5 days.

1×LiAc/TE: 1×LiAc, 1×TE

1× PEG/LiAc/TE: 40% PEG 4000, 1×LiAc, 1×TE

10×TE: 0.1 M Tris-HCl (pH7.5), 0.1 M EDTA

10×LiAc: 1 M lithium acetate (Sigma #L-6883), pH7.5.

2.2.5.6 Yeast mating

Yeast mating is an alternative method to verify positive two-hybrid interactors by introducing two different plasmids into the same host cells. The reporter genes will be activated only in the presence of bait protein and prey protein.

RSI fused to the GAL4 DNA binding domain vector was transformed into Y187 (MAT**a**), and all putative RSI interactors fused to the GAL4 activation domain vector were transformed into Y190 (MATα). The two haploid yeast strains were then mated for 6-8 hrs at 30°C and spread on selective media ($Trp^-His^-Leu^-$). After 3-5days, diploid cells were tested for β-galactosidase activity using the filter assay.

2.2.5.7 Preparation of yeast competent cells

A single yeast colony which was grown on an appropriate selective YSD media (-T) was transferred into 25 ml of the selective YSD liquid media (-T). The overnight culture was used to inoculate a 500 ml of the YPD media (OD_{600} was around 0.2) and grown till OD_{600} reached to 0.4-0.5. This took approximately 4-4.5 hrs. If the cells had visibly clumped, they were dispersed with gently vortexing before using them in the next step. The cells were collected by centrifugation at 2,000 g for 5 mim, washed once with 1/10 volume of distilled water and 1×LiAc/TE. The cells were resuspended in the YSD media without 10× dropout and used for library screening.

2.2.5.8 Preparation of carrier DNA

High efficiency transformation of yeast cells uses single stranded nucleic acids as a carrier (Schiestl and Gietz, 1989). Salmon testis DNA (Sigma-D1626 Type III Sodium from Salmon testis) was dissolved in TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA), 10 mg/ml, by pipetting the solution. It was then incubated overnight at 4°C to give a homogenous viscous solution. Thereafter, it was sonicated twice for 30 s with a large probe at ¾ power. The resulting DNA had an average size of 7 kb as judged from an ethidium bromide gel, ranging in size from 2 to 15 kb. Too strong sonication, leading to carrier DNA with smaller average size of about 1-2 kb, dramatically reduced the transformation efficiency.

The DNA solution was extracted once with TE-saturated phenol, once with TE-saturated phenol:chloroform (1:1), and once with chloroform. The DNA was then percipitated by adding 1/10 volume of 3 M sodium acetate (pH 6.0) and 2.5 volume of ice cold ethanol (99.9%). The precipitate was collected by centrifugation at 12,000 g and washed with 70% ethanol, partially dried under vacuum and redissolved in TE at 10 mg/ml. The DNA was denatured in a boiling water bath for 20 min, then immediately cooled in an ice water bath. The DNA solution was stored in aliquots at -70°C and thawed when needed.

2.2.6 *In planta* transformation of *Arabidopsis thaliana* by vacuum infiltration

2.2.6.1 Growth of plants for transformation (T_0)

Seeds were germinated in a community tray in a short day (SD) growth chamber (9 hrs light, 22°C). Three weeks after sowing, seedlings were transferred into 10 cm pots. The plants were further grown for two more weeks. If desired, it was possible to keep the plants longer under SD for up to 2 to 3 more weeks. Thereafter, they were transferred to long day (LD) conditions (16 hrs light, 22 °C) to promote bolting. Once the primary inflorescence shoots reached about 10 cm (approx. 2 to 3 weeks later), they were removed in order to induce the development of rosette inflorescence shoots. The plants were ready for infiltration 6 to 7 days later.

2.2.6.2 Growth of *Agrobacterium*

The T-DNA constructs were made in the pBIN19 binary vector system (Frisch *et al*., 1995). The vector carrying the BAR gene was generally used because it allowed a more reliable selection of transformants. Binary vectors were transformed by electroporation into the *Agrobacterium* strain GV3101 (pMP90) (Koncz and Schell, 1986). A 25 ml liquid culture (YEB + antibiotics) was started from a single colony and grown at 28 °C for 2 days. The day before the infiltration (Becker *et al*., 1992), 10 ml of this preculture was used to inoculate 500 ml medium (YEB+antibiotics) and incubated overnight. On the following day, the cells were harvested by centrifugation at 4000 g for 15 min and resuspended in infiltration medium (IM) to an OD between 0.8 – 1.2. The bacterial cells were routinely resuspended in 1 l of IM.

YEB medium/l: 5 g of Gibco beef extract, 1 g of bacto yeast extract, 1 g of bacto tryptone, 5 g of sucrose pH 7.4, and add 2 ml of 1 M $MgSO_4$ after autoclaving

Antibiotics for GV3103 (pMP90) +Kan^R binary vector: 100 mg/ml Rifampicin, 25 mg/ml of Gentamycin, 50 mg/ml of Kanamycin

Infiltration medium (IM): 0.5× MS salts, 1× B5 vitamins, 5% sucrose, 0.044 µM benzylaminopurine (10 µl/l of a 1 mg/ml stock), pH 5.7 (with KOH). After autoclaving and prior to use, add BAP and the surfactant SILWET L-77 (Osi Specialties, Inc; 0.005% v/v-50 µl/l).

2.2.6.3 Vacuum infiltration and selection of transformants

A 30 cm diameter glass bell jar was connected via a condensation trap to a Leybold Trivac oil pump (type S 8B/AF 4-8). To the bell jar 2 glass trays (29 cm×10 cm×5 cm) were fit, each containing 500 ml of the *Agrobacterium* suspension. Each tray could hold two inverted pots. Only the inflorescence shoots were submerged in the *Agrobacterium* suspension. During the treatment under vacuum of about 16 mbar for 5 min, the suspension bubbles profusely. After the treatment the vacuum was released quickly by removing the rubber tubing that connects the bell jar to the condensation trap. The bacterial suspension was reused for the next plants. After the infiltration treatment the plants looked somewhat glassy. Plants were taken back to a LD growth chamber or to the greenhouse, the temperature was kept lower than 25°C. Under this condition, plants recovered quickly, and one day after the infiltration treatment already displayed normal newly-opened flowers.

The seeds were collected in paper bags. Bagged plants were watered for one more week and then allowed to dry for 1 to 2 more weeks. Hundreds of seeds were distributed in several 12 cm pots. Transgenic plants were selected by spraying with a BASTA (herbicide) solution (0.1% for one-two week old plants, 0.5% for two-three week old plants).

2.2.7 Methods for i*n situ* hybridization in Planta

2.2.7.1 *In situ* hybridization with digoxigenin (DIG) labeled probes

In Situ hybridization techniques (Coen *et al*., 1990) allow to detect spatial and temporal expression of specific genes in morphologically preserved plant tissue sections. The DIG labeling method is based on a steroid isolated from digitalis plants (*Digitalis purpurea* and *Digitalis lanata*). As the blossoms and leaves of these plants are the only natural source of digoxigenin, the anti-DIG antibody does not bind to other biological material.

2.2.7.1.1 Size of tissue and penetration of fixative

Plant tissues were fixed immediately after dissection in 4% formaldehyde. In general, the smaller the size of tissues is the better results of *in situ* hybridization. Plant tissues were dissected using a razor blade. Before use, any fat or grease was removed from the blade using 100% ethanol because of the fat might seal the tissue thus infiltration of the fixative is

prevented. The tissues were cut as small as possible on a clean glass-plate. One dimension of the tissue was < 1mm, and the others was < 10mm.

To preserve the morphology, the biological material was fixed using 4% formaldehyde. Plant tissues contain air between the cells, therefore they float on the surface of the fixative. For the better penetration of the fixative, the tissues were vacuum infiltrated for a few minutes. Because formaldehyde vapor is volative, the fixative was replaced after the vacuum treatment.

2.2.7.1.2 The procedure of fixation (Day 1), dehydration steps (Day 2-4), wax embedding (Day 4-8)

Fixation is one of the most critical steps for successful *in situ* hybridization. Poorly fixed material will give little or no *in situ* signal even with probes for highly abundant mRNAs. Fixation should provide RNA retention while allowing accessibility of the probe to the RNA.

After fixation, the tissue was washed and subsequently dehydrated in an ethanol series. Replacement of fresh paraffin wax (melted at 60°C) was done to get rid of any traces of histoclear.

Day 1

4% paraformaldehyde: 10× PBS and paraformaldehyde (stored in a cold room)

Method: Prepare PBS using a graduate cylinder, pour into a sterile flask, add 2-3 pellets of NaOH, microwave up to 60 °C. Weigh paraformaldehyde in fume hood, add to PBS, seal flask with parafilm, shake until dissolved, put flask on ice. Adjust pH to 7.0 with H_2SO_4. Paraformaldehyde is very toxic, always seal well or keep in fume hood.

Day 2

0.85% saline	30 mins	on ice
50% ethanol/0.85% saline	3 hrs	on ice
70% ethanol/0.85% saline	3 hrs	on ice
85% ethanol/0.85% saline	overnight	4°C

Day 3

95% ethanol/dH_2O	4 hrs	4°C
100% ethanol/dH_2O	4 hrs	4°C

100% ethanol/dH_2O	overnight	4°C

Day 4

100% ethanol	2 hrs	room temp
50% ethanol/50% histoclear	1 hr	room temp
100% histoclear	1 hr	room temp
100% histoclear	1 hr	room temp
100% histoclear	1 hr	room temp

50% histoclear/50% wax chippings, overnight at 58°C

Day 5-7

The 50% histoclear/50% wax chippings was replaced with 100% wax and changed twice daily. The wax was poured slowly to avoid formation of bubbles.

Day 8

The tissue blocks was made using 15 cm Petri dish. A layer or wax was poured sufficient to cover the tissue and to orientate the tissue in the mold using preheated forceps. Tissue blocks was kept molten in a 58°C incubator for a while. They were solidified on ice and waited till wax just begins to come solid, then the mould floated on water. After the surface began to solidify and indent, the mould was inverted and left for 5 min to solidify completely. The blocks were stored at 4°C until used.

2.2.7.1.3 Sectioning

The block was cut to a trapezoid shape, leaving about 2 mm of wax around the tissue. The ribbons of section was cut at 8 µm thickness, floated onto sterile water on coated slides then placed on a 42°C hotplate for a few minutes until the ribbon flattened out. The excess water was drained off and then tissues pressed with lens. Sections were left on the hotplate overnight to dry.

2.2.7.1.4 Probe preparation

To obtain a specific probe, the DNA fragment was subcloned into a vector containing T3 or T7 RNA polymerase promoters. The insert, together with T3 or T7 RNA polymerase binding sequences, was amplified by PCR, gel purified by using the agarose gel extraction kit and eluted in DEPC-treated water to give a concentration of 100-200 ng/µl.

2.2.7.1.5 Procedure for digoxigenin labeling of probes

The reaction mixture was incubated for 2 hrs at 37°C. From the reaction mixture, 1 µl was taken and tested on agarose gel. To the rest of reaction mixture, 75 µl of 1× MS buffer, 4 µl of tRNA (50 mg/ml) and 2 µl of DNAse (Boehringer) were added and incubated further for 15 min at 37°C. The mixture was checked again on agarose gel by loading 2 µl of it. To the mixture 100 µl of 3.8 M NH_4Ac, 600 µl of ice-cold absolute EtOH was added and left overnight. On the following day it was centrifuged down at 13,000g (14,000 rpm in a HERAEUS #3754 roter), washed with 80% EtOH, air-dried and resuspended in 50 µl of DEPC-treated water. The concentration of the probe was determined before hybridization following the manual provided by the Boehringer DNA labeling kit.

Reaction mixture:

template	2 µl
Dig-UTP mix	2 µl
transcription buffer	2 µl
RNA polymerase (T3 or T7)	2 µl
DEPC-water	up to 20 µl total volume

1×MS buffer: 10 mM Tris-HCl pH 7.5, 10 mM $NaCl_2$, 50 mM NaCl.

2.2.7.2 *In situ* hybridization

2.2.7.2.1 Tissue pretreatment

The procedures of tissue pretreatment were performed using a glass cylinder rinsed with autoclaved water and with the sterilized solution using autoclaved distilled water. All solutions were poured in glass troughs. The slides were put in stainless steel racks and passed through the following series of solutions.

1. Histoclear	10 min
2. Histoclear	10 min
3. 100% EtOH	1 min
4. 100% EtOH	30 sec
5. 95%EtOH	30 sec

6. 85%EtOH, 0.85% saline	30 sec
7. 50%EtOH, 0.85% saline	30 sec
8. 30%EtOH, 0.85% saline	30sec
9. 0.85% saline	2 min
10. PBS in 2×SSPE	20 min at 70°C
11. PBS	2 min
12. Pronase in 1× Pronase buffer	10 min
13. Glycine (0.2% in PBS)	10 min
14. PBS	2 min
15. Paraformaldehyde	10 min
16. PBS	2 min
17. PBS	2 min
18. 0.85% saline	2 min

19. Dehydrated through the ethanol series from No. 8 to 4 in a reverse way

10× PBS: 1.3 M NaCl, 0.03 M Na_2HPO_4, 0.03 M NaH_2PO_4

Pronase (Sigma P-6911): 40 mg/ml in dH_2O. Predigested by incubating for 4hrs at 37°C. Stored in 1 ml aliquots at -20°C

20× pronase buffer: 1 M Tris-HCl, pH 7.5, 0.1 M EDTA

10% Glycine: 10 g in 100 ml of dH_2O. Stored at 4°C.

2.2.7.2.2 Hybridization

The following hybridization mixture was prepared, spun down shortly and left at room temperature till it is needed. The hybridization mixture was prepared by mixing 2 µl of probe, 2 µl of dH_2O, 4 µl of formamide. The riboprobes were commonly used at a concentration of 0.1-0.3 ng/µl/kb. The probe was denatured for 2 min at 80°C, spun down and cooled on ice. Then the probe was mixed with the hybridization buffer (32 µl/slide) and left at room temperature. The slides were taken out from a pretreatment solution, allowed to dry completely. Per slide 40 µl of hybridization mix was used. To keep the hybridization tray humid, a paper

towel was soaked with 2× SSC, 50% formamide and placed to the bottom of the tray. Hybridization was performed in a chamber at 50°C overnight.

Hybridization buffer for 24 slides:

10× salts	100 µl
Deionized formamide	400 µl
tRNA 50mg/ml	10 µl
100× Denhardt's solution	10 µl
H_2O	80 µl
50% dextran sulphate	200 µl
Final volume	800 µl

2.2.7.2.3 Hybridization washes

Before starting the washing step, two water bathes were set at 37°C and 50°C. Slides hybridized overnight were put back into stainless steel racks, placed in a trough containing the washing buffer (2× SSC, 50% formamide) and left for 30 min at 50°C. The washing buffer was replaced with a new one, and slides were incubated further for 1.5 hrs at 50°C. The slides were washed again with the 1× NTE buffer two times for 5 min at 37°C. The racks containing slides were incubated in the NTE buffer containing 20 µg/ml RNase A for 30 min at 37°C. After the incubation with RNase A, the slides were washed step by step: two times for 5 min at RT with NTE buffer, 1 hr at 50°C with the washing buffer, 2 min at RT with 1× SSC and 5 min at RT with PBS. In the final washing step with PBS, the slides were left at 4°C overnight.

10× NTE buffer: 5 M NaCl, 100 mM Tris-HCl pH 7.5, 10 mM EDTA.

2.2.7.2.4 Antibody staining

For the antibody staining, a 15 cm square Petri dish was used. It contained 5 slides in 20 ml of buffers used in series:

Bufferr 1	5 min
Buffer 2	1 hr
Buffer 3	30 min
Buffer 4	1.5 hrs

Then the slides were washed with buffer 3 four times each time for 20 min, equilibrated in buffer 1 for 5 min and in buffer 5 for 5 min. The slides were incubated with buffer 6 for 1 – 2 days in the dark. The expression signal was checked after incubation for 12 hrs. According to the intensity of the signal and the background, additional dehydration washing was performed. For counter-staining the slides were incubated with 0.1% of calcofluor for 5 min and washed briefly with water. The slides were air-dried and covered with coverslips after dropping Entellan (Sigma). The slides were inspected with a light microscope.

Buffer 1: 100 mM Tris-HCl, 150 mM NaCl

Buffer 2: 0.5% blocking reagent (Boehringer provided) in buffer 1. It was made freshly. Dissolved at 60-70°C for 1 hr, the solution remained turbid

Buffer 3: 1% BSA, 0.3% Triton X-100 in buffer 1

Buffer 4: Anti-digoxigenin-AP 1:30000 in buffer 3

Buffer 5: 100 mM Tris-HCl, 100 mM NaCl, 50 mM $MgCl_2$, pH 9.5

Buffer 6: 90 µl of NBT, 90 µl of BCIP in 45 ml of Buffer 5.

2.3 Genetic nomenclature

In this dissertation, genotypes are written in italics with the wild-type genotype in capitals (e.g., *AmGRAS*) and the mutant genotype is lowercase letters (e.g., *amgras*). Wild-type polypeptide gene products are written in nonitalic capital letters (e.g., AmGARS). Double mutant genotypes are written in italic lowercase letter (e.g., *rga-24 gai-t6*).

3. Results

3.1 Isolation of RSI interacting partners using the yeast two-hybrid system

RSI was isolated as a putative regulator of *DEF* gene activity based on the spatial and temporal complementary expression pattern. In later floral developmental stages, there is no transcript of *DEF* found in sporogenous tissue of stamens, in contrast to the presence of *RSI* transcripts. To test whether RSI requires other factors to regulate the spatial and temporal expression of *DEF*, a GAL4-based yeast two-hybrid system with two reporter genes, *HIS3* and *LacZ* , was utilized.

The yeast two-hybrid system is based on the fact that many eukaryotic transcriptional activators have at least two domains which are physically separable and functionally independent. The yeast GAL4 transcription factor contains a DNA-binding domain and a transcriptional activation domain (Keegan *et al*., 1986; Hope and Struhl, 1986; Ma and Ptashne, 1987). If a protein fused to the GAL4 DNA-binding domain (the bait) interacts with a protein fused to the GAL4 activation domain (the prey), then the reporter gene is activated (Fields, 1989).

RSI cannot activate transcription on its own in different yeast genetic backgrounds which was confirmed by testing it for reporter gene activation in the pGBT9 binding domain vector. Therefore, it could be used as a bait to screen an *Antirrhinum* cDNA expression library.

In the first screen of approximately 4,000,000 recombinant yeast colonies, three independent positive ($His3^+$, Trp^+, Leu^+ and $LacZ^+$) candidates (Y6, Y13 and Y35) were identified. In the second screen of 12,000,000 recombinant yeast colonies, five times Y6, three times Y35 and additionally four independent clones (2Y6, 2Y14, 2Y16 and 2Y19) were rescued.

All positive clones were further tested for histidine prototrophy with RSI in three different yeast genetic backgrounds, together with a negative control construct (pGBT9 and pLAM5') which was provided by CLONTECH®. In addition, β-galactosidase filter assay was used to demonstrate the interaction of RSI with these proteins in yeast (Fig. 3).

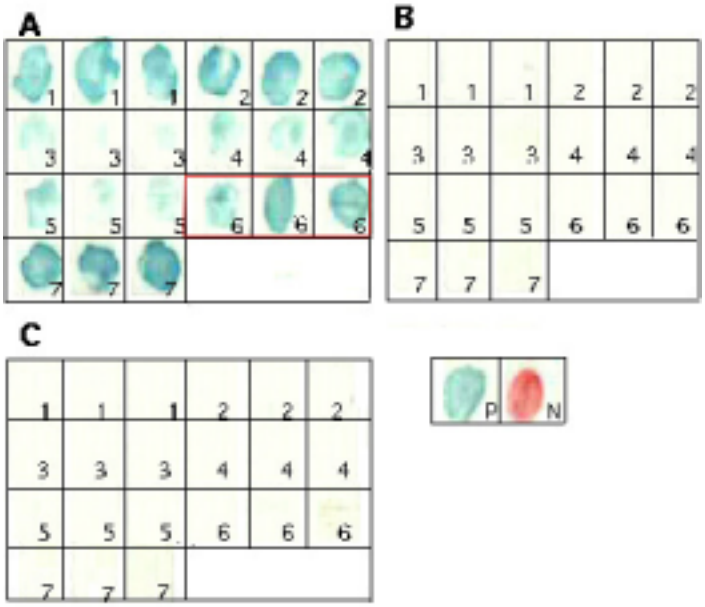

Fig. 3. β-Gal filter assay of seven putative RSI interactors.

Different combinations of bait and activation domain constructs were co-transformed into the yeast strain SFY526, grown for 3 days on filters and tested for blue colour staining after being permeabilized by liquid nitrogen.

A: full length RSI containing vector (RfpGBT9) + putative RSI interactors in pGAD424.

B: empty binding domain vector (pGBT9) + putative RSI interactors in pGAD424.

C: lamin protein containing vector (pLAM5') + putative RSI interactors in pGAD424.

No. 1=Y6, No. 2=Y35, No. 3=Y13, No. 4=2Y6, No. 5=2Y14, No. 6=2Y16, No. 7=2Y19.

P: DEF in pGBT9 + GLO in pGAD424 as positive control.

N: RSI in pGBT9 + empty pGAD424 as negative control.

All seven putative RSI interactors were co-transformed into the yeast strain SFY526 for the β-galactosidase filter assay. The co-transformation was repeated three times with the full length RSI construct (RfpGBT9, Fig. 3,A), the 'empty' binding domain vector pGBT9 (Fig. 3,B) and the lamin containing construct pLAM5' (Fig. 3, C). All co-transformed yeast clones were grown on SD selective (-Trp ,-Leu) media and tested for *LacZ* gene expression.

To quantify the strength of interaction, β-gal liquid assays were performed and the β-galactosidase enzymatic activity was calculated (Fig. 4).

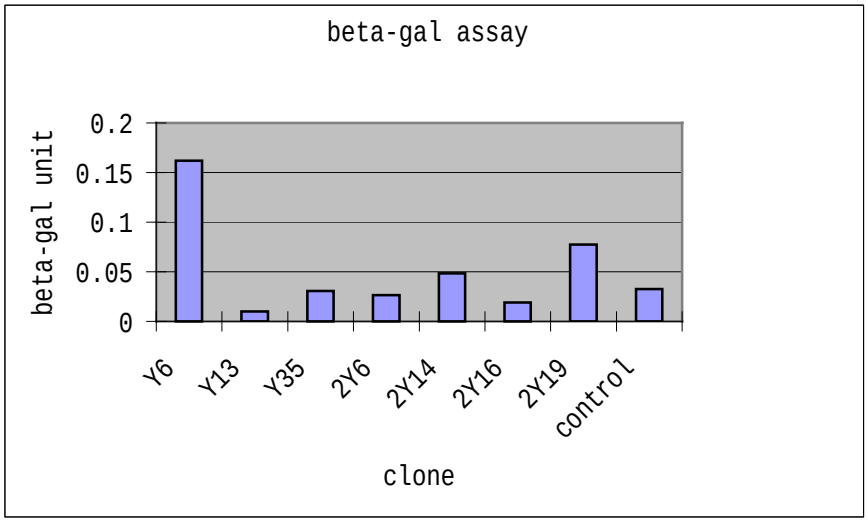

Fig.4. β-galactosidase enzymatic activity of seven RSI interactors. β-gal units were calculated by the formula, $(1000\times A_{420})/T\times V\times A_{600}$. The histogram table is displaying the average of β-gal activity from the two different yeast strains, Y190 and SFY526. Seven interactors were co-transformed with the RSI, bait construct. These yeast cells were incubated in YSD selective liquid media (-Trp and -Leu) at 30°C overnight and next day the cultures were transferred into YPD liquid media to quantify the β-galactosidase activity. DEF in pGBT9 as bait + GLO in pGAD424 as prey was used as a positive control.

Y6 (No.1 in Fig.3) and 2Y19 (No. 7 in Fig. 3) showed strong *LacZ* expression in the presence of RSI. Whereas Y13 (No. 3 in Fig. 3) showed only weak *LacZ* activity in the liquid and solid β-galactosidase assay, suggesting that the interaction with RSI is either weak or of transient nature. 2Y16 (later renamed AmGRAS) is highlighted with a red box, because this clone was of prime interest in the following work.

3.2 Verification of protein-protein interaction with RSI using yeast mating

As an independent method to confirm protein-protein interaction *in vivo*, yeast mating was utilized. With this method, the protein fused to the GAL4 DNA binding domain vector (the bait) and the protein fused to the activation domain vector (the prey) are expressed in two different haploid yeast strains of opposite mating type (**a** and α). The strains were mated using a replica-plating tool to determine whether two proteins are interacting. Only mated diploid cells grow on selective media. Mated cells were further tested for *LacZ* gene expression using the β-gal filter assay (Fig. 5).

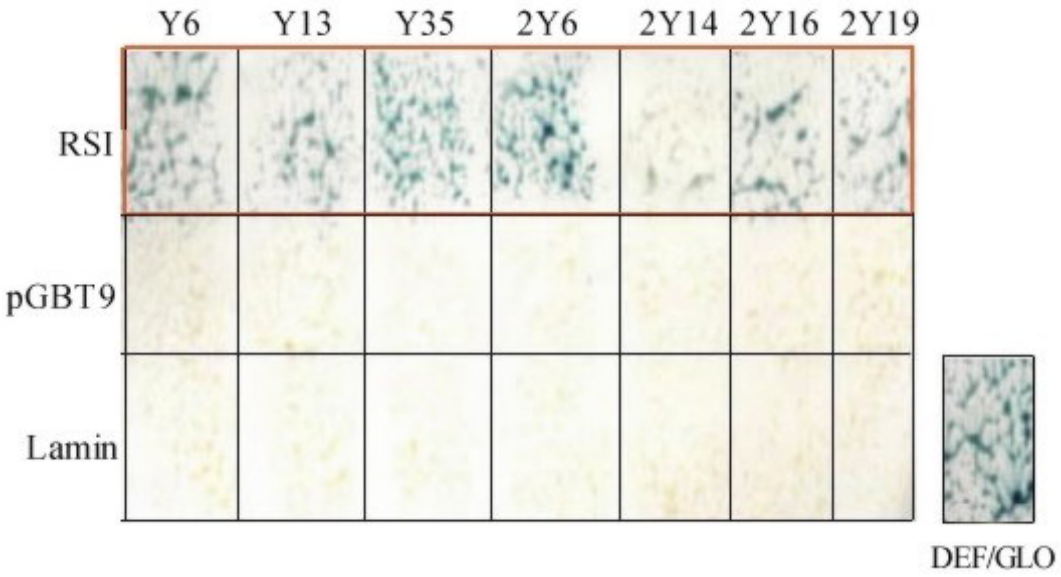

Fig. 5. Yeast mating with RSI and seven putative RSI interactors.
RSI, pGBT9 (empty binding domain vector) and Lamin (lamin protein in pLAM5'vector) were transformed into yeast strain Y187 (MAT**a**). These proteins are depicted in a left column lines. All individual seven putative RSI interactors (in a top line) were transformed into yeast strain Y190 (MATα). Independently grown two haploid yeast strains (depicted above in rows and columns) were mated using a replica-plating tool covered with sterile velvet, and were grown on a selective media ($Trp^{-}His^{-}Leu^{-}$) for 2 days. Diploid cells were transferred into sterile filter and tested for the β-galactosidase activity. As a positive control DEF/GLO proteins, expressed in the appropriate vectors, have been used.

3.3 Possible functions of putative RSI interactors deduced by sequence comparison

To gain insight into the nature of these clones, BLAST searches were performed to identify similar proteins from different organisms in various databases (Table 1). In the following each clone (a total of seven obtained from the yeast two-hybrid screen) will be described individually. In addition, GST pull-down assays to confirm the interaction in an *in vitro* system were carried out.

Clone	LacZ expres-sion *in vivo*	Self-activation	*In vitro* GST pull-down	Amino acid sequence similarity	Data bank No.	Organism	E-value Positives (Identities) %
Y6	**yes**	**yes**	**positive**	Hypothetical protein Unknown protein KIAA0775 gene product	AL132964 AB016881 AB018318	*Arabidopsis* *Arabidopsis* *Homo sapiens*	62(41) 60(36) 52(32)
Y13	**yes**	**yes**	**positive**	**Ser/Thr kinase**	BAB02869	*Arabidopsis*	**87(70)**
Y35	**yes**	**yes**	*****	**Hypothetical protein**	AAD19768	*Arabidopsis*	**89(85)**
2Y6	**yes**	*	**positive**	Hypothetical protein Unknown protein Hypothetical protein	AL133421 AB006696 AE000718	*Arabidopsis* *Arabidopsis* *Aquifex aeolicus*	61(38) 59(37) 44(23)
2Y14	**yes**	**no**	**positive**	Centromere protein Probable centromere protein RHO-associated coiled-coil forming kinase	AB022223 AL161539 U58513	*Arabidopsis* *Arabidopsis* *Mus musculus*	61(41) 57(37) 49(29)
2Y16	**yes**	**yes**	**positive**	**RGA** **GAI** **SCR**	AJ224957 AL161539 U58513	*Arabidopsis* *Arabidopsis* *Arabidopsis*	**68(50)** **69(48)** **40(25)**
2Y19	**yes**	**no**	**positive**	Putative N-methyl transferase Hypothetical protein	Y16952 NC_002696	*Amycolatop-sis* *Caulobacter*	53(24) 48(33)

Table 1. Putative interacting partners of RSI.

Amino acid sequence similarity and the percentage of Expected (E)-value have been obtained using the BLAST search program.

The Expect value (E) is a parameter that describes the number of hits one can "expect" to see just by chance when searching a database of a particular size. It decreases exponentially with the Score (S) that is assigned to a match between two sequences. Essentially, the E value describes the random background noise that exists for matches between sequences. Positives: the number of hits of similar amino acids, Identities: the number of hits of identical amino acids

Highly scored protein sequences are bolded. * : not tested.

3.3.1 Clone Y6

DNA sequencing revealed that Y6 encodes a protein of 104 amino acids. This protein was rescued five times from the yeast two-hybrid screening and showed sequence similarity with a hypothetical protein from *Arabidopsis*, the function of which is unknown (Table. 1). The Y6 protein contains a highly conserved acidic domain. It turned out to be self-activating, because if the Y6 ORF (Open Reading Frame) was swapped from the pGAD424 activation domain vector (AD) into the pGBT9 DNA binding domain (BD) vector, it could activate the reporter gene on its own. Therefore, swapping the Y6 ORF was not feasible to test the interaction in the other direction. However, if RSI in the BD vector and Y6 in the AD vector are expressed together, there is transcriptional activation of the reporter gene.

The data bank search revealed three proteins from *Arabidopsis* with homology to clone Y6, the function of which is not known.

The alignment of Y6 with the three hypothetical, acid domain containing proteins is shown in Fig. 5.

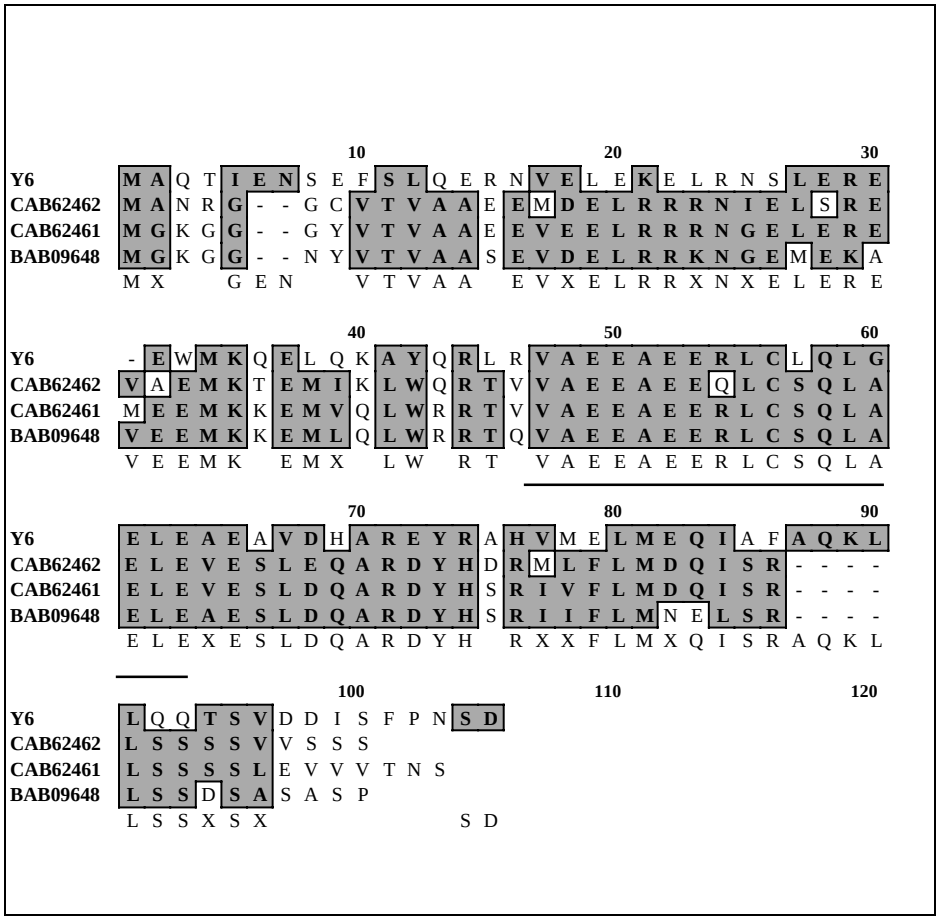

Fig. 5. Amino acid sequence alignment of and the three unknown proteins (CAB62462, CAB62461 and BAB09648) from *Arabidopsis*. Identical residues, conserved between Y6 and the other unknown proteins, are displayed in reverse type, and similar residues are in gray boxes. Gaps introduced to improve the alignment are indicated by blanks and sequence truncations by dashes. The highly conserved acidic domain is underlined.

3.3.1.1 *In-vitro* GST–Y6 fusion protein pull-down assay

As an independent method to confirm the protein-protein interaction, an *in vitro* GST pull-down assay was carried out. The principle of the GST fusion protein pull-down assay is to use the affinity of GST for glutathione-coupled beads to purify complexes of interacting proteins from a solution of non interacting proteins, in order to confirm suspected interactions (modified

from Bhalerao *et al*., 1999).

A PCR generated EcoRI-XhoI cDNA fragment containing the Y6 ORF from the pGAD424 clone was cloned into the pGEX-5X-1 vector and used for the purification of the glutathione S-transferase (GST)-fusion protein. The EcoRI-XhoI cDNA fragment was generated using primer 5AD (contains an EcoRI site) and primer y6Xho-3 introducing a XhoI site at the 3'-end of the PCR fragment.

The GST-Y6 fusion protein was expressed and purified from bacteria. To prepare a cell lysate, cDNA fragment of RSI (obtained from Dr. M. Roccaro) served as a template to synthesize [^{35}S] methionine-labeled RSI protein in a TNT® Coupled Reticulocyte Lysate Systems (Promega). The GST-Y6 fusion protein and the RSI cell lysate were mixed in the presence of glutathione-agarose beads and incubated to allow protein association to occur. The GST-Y6 fusion protein and associated proteins were collected by centrifugation and the complexes were washed. The protein complexes were eluted from the beads with excess free glutathione and separated on a 10% SDS-polyacrylamide gel to detect ^{35}S-labeled RSI protein by autoradiography (Fig. 6).

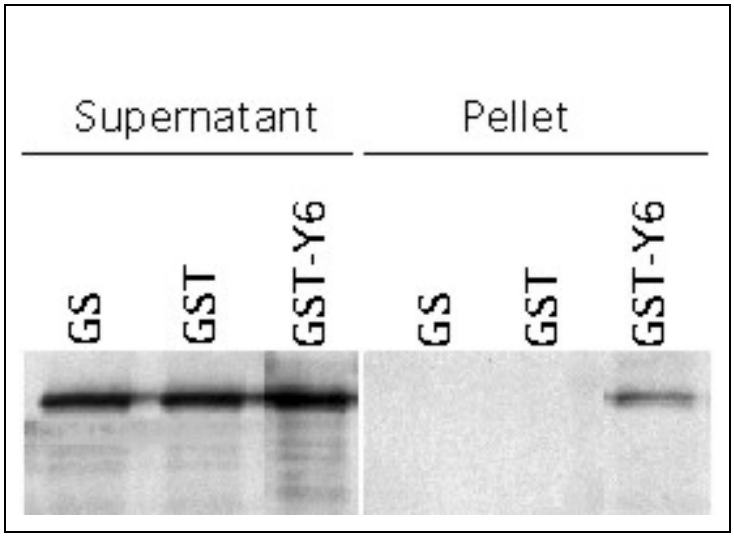

Fig. 6. *In-vitro* GST-Y6 fusion protein pull-down assay with ^{35}S-labeled RSI. The empty matrix (GS), control GST and GST-Y6 (10 µg of each) bound to glutathione-agarose were incubated with 2.5 µl of *in vitro* translated ^{35}S-labeled RSI using TNT® Coupled Reticulocyte Lysate Systems. Proteins bound specifically to glutathione agarose, together with the supernatant fraction, were separated on a 10% SDS-polyacrylamide gel to detect the ^{35}S-labeled RSI protein by autoradiography. The film was exposed for 5-6 days at -70°C.
Empty matrix (GS): glutathione-agarose beads, control GST: glutathione S-transferase (GST) protein, and GST-Y6: GST-Y6 fusion protein.

3.3.1.2 Summary of clone Y6 features

Y6 displays homology to three *Arabidopsis* proteins with unknown function. Y6 interacts with RSI in the yeast two-hybrid system and also *in vitro*, as judged from a GST pull-down experiment. Its function so far is unknown.

3.3.2 Clone Y13

The partial Y13 clone which was rescued as a 1500 bp fragment showed 70% identity with a Ser/Thr protein kinase from *Arabidopsis*. The β-galactosidase filter assay shows that the interaction of Y13 with RSI is weak which is a typical feature of kinase/ substrate interactions

(Fig.3). The swapping of Y13 from the AD vector to the BD vector showed self-activation of the reporter gene, indicating that Y13 contains a transcription activation domain.

Alignment of the partial Y13 protein with the Ser/Thr protein kinase (BAB02869) shows the presence of highly conserved stretches of amino acids (Fig. 7).

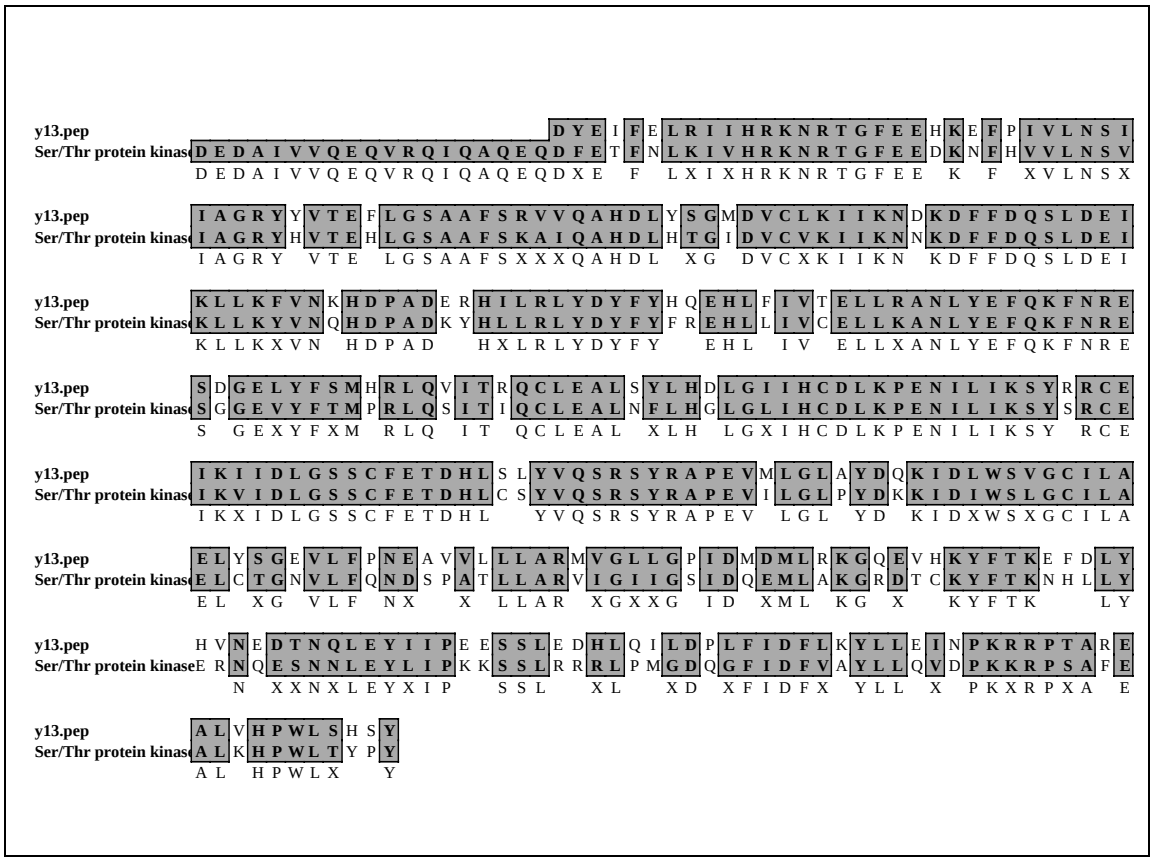

Fig.7. Amino acid sequence comparison of Y13 and to the Ser/Thr protein kinase (BAB02869) from *Arabidopsis*. Identical residues conserved between Y13 and the Ser/Thr protein kinase are displayed in reverse type, and similar residues are in gray boxes. Gaps introduced to improve the alignment are indicated by blanks and sequence truncations by dashes.

3.3.2.1 *In-vitro* GST–Y13 fusion protein pull-down assay

A PCR generated EcoRI-SmaI cDNA fragment containing the Y13 ORF was used for purification of the glutathione S-transferase (GST)-fusion protein. The EcoRI-SmaI cDNA fragment was generated using primer 5AD and primer y13Sma-3 (introducing a SmaI site in the 3' end). The experiment was carried out as described for clone Y6. ^{35}S-labeled RSI protein was detected from the matrix-bound proteins by autoradiography (Fig.8).

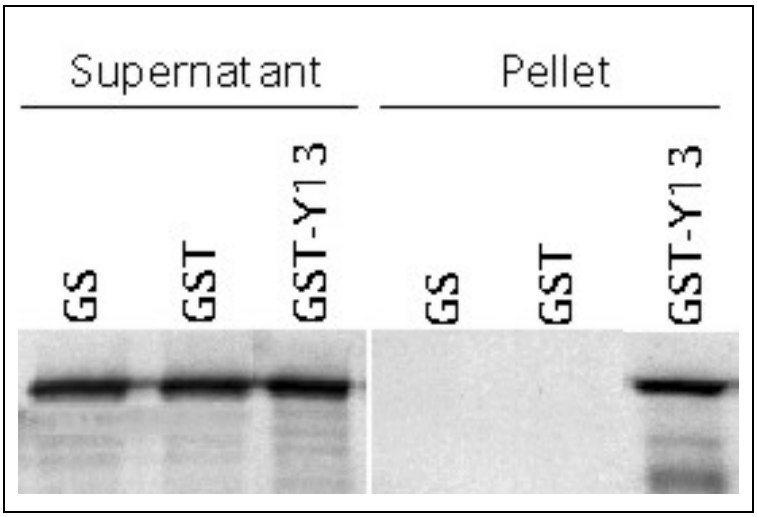

Fig. 8. *In-vitro* GST-Y13 fusion protein pull-down assay with ^{35}S-labeled RSI. The empty matrix (GS), control GST and GST-Y13 (10 µg of each) bound to glutathione-agarose were incubated with 2.5 µl of *in vitro* translated ^{35}S-labeled RSI using TNT® Coupled Reticulocyte Lysate Systems. The procedure of separation and detection of proteins followed that of clone Y6.
Empty matrix (GS): glutathione-agarose beads, control GST: glutathione S-transferase (GST) protein, and GST-Y13: GST-Y13 fusion protein.

3.3.2.2 Summary of clone Y13 characteristics

Y13 displays homology to a Ser/Thr protein kinase (BAB02869) from *Arabidopsis*. Y13 interacts with RSI in the yeast two-hybrid system and also *in vitro*, as judged from a GST pull-

down experiment.

3.3.3 Clone Y35

Y35 showed 85% amino acid identity with a hypothetical protein (AAD19768) of unknown function from *Arabidopsis*. The alignment of this clone with the hypothetical protein is shown in Fig. 9.

In vitro GST pull down assay was not performed for this protein.

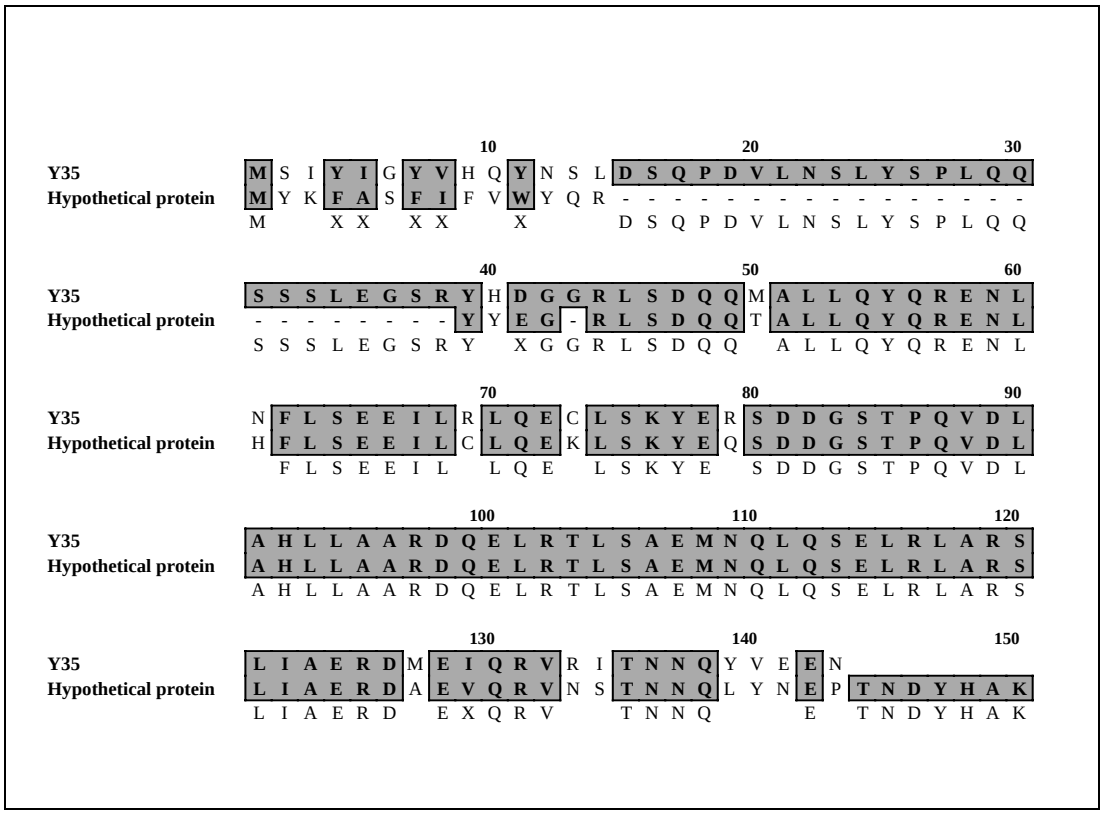

Fig. 9. Amino acid sequence comparison of Y35 with the hypothetical protein (AAD19768) from *Arabidopsis*. Only the highly conserved region between Y35 and the protein from *Arabidopsis* is aligned. Identical residues conserved between Y35 and the hypothetical protein are displayed in reverse type, and similar residues are in gray boxes. Gaps introduced to improve the alignment are indicated by blanks and sequence truncations by dashes.

3.3.4 Clone 2Y6

2Y6 was rescued as a 1500 bp long fragment. It showed high sequence similarity with hypothetical proteins from *Arabidopsis*, the function of which are unknown (Table 1).

A database search with Y6 detected similarity to a conserved domain of a 116KDa subfamily of V-type ATPases from bacteria, which plays a role in proton transport and assembly of the V-type ATPase complex. In yeast, its subunits are encoded by two homologous genes, *VPH1* and *STV1*.

Vector swapping was not performed, therefore it is not known whether this protein contains a transcription activation domain.

3.3.4.1 *In-vitro* GST–2Y6 fusion protein pull-down assay

A PCR generated EcoRI-SmaI cDNA fragment was used for purification of glutathione S-transferase (GST)-fusion protein. The EcoRI-SmaI cDNA fragment was generated using primer 5AD and 2y6Sma-3 primer (introducing a SmaI site in the 3' end).

The GST pull-down experiment was performed as described before. ^{35}S-labeled RSI protein was detected by autoradiography (Fig. 10).

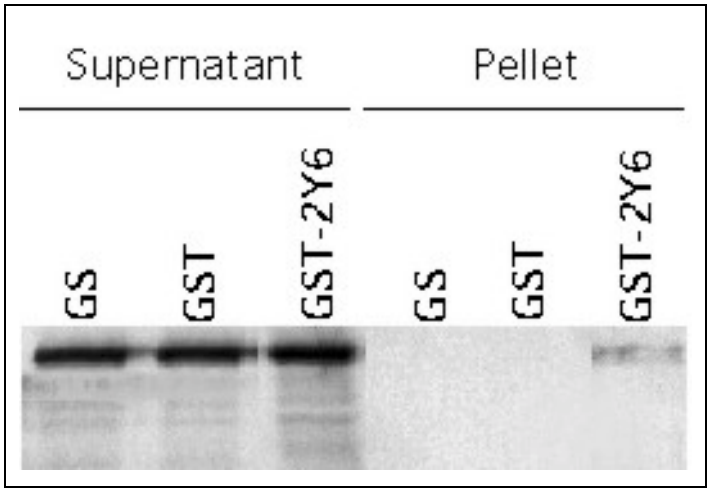

Fig.10. *In-vitro* GST-2Y6 fusion protein pull-down assay with ^{35}S-labeled RSI. The empty matrix (GS), control GST and GST-2Y6 (10 µg of each) bound to glutathione-agarose were incubated with 2.5 µl of *in vitro* translated ^{35}S-labeled RSI using TNT® Coupled Reticulocyte Lysate Systems. The separation and detection of protein followed the way of clone Y6.
Empty matrix (GS): glutathione-agarose beads, control GST: glutathione S-transferase (GST) protein, and GST-2Y6: GST-2Y6 fusion protein.

3.3.4.2 Summary of features of clone 2Y6

2Y6 displays homology to unknown proteins from *Arabidopsis* and *Aquifex aeolicus*. 2Y6 interacts with RSI in the yeast two-hybrid system and also *in vitro*, as judged from a GST pull-down experiment.

3.3.5 Clone 2Y14

2Y14 was rescued as a 1100 bp long fragment. It showed sequence similarity with a centromere protein homolog from *Arabidopsis* and with a Rho-associated coiled-coil containing protein kinase p160 ROCK-2 from *Mus musculus* (Table 1). This protein did not show self-activation upon swapping to the BD vector. Therefore the interaction with RSI could be confirmed further via co-transformation with 2Y14 in the BD vector and RSI in the AD

vector.

3.3.5.1 *In-vitro* GST–2Y14 fusion protein pull-down assay

A PCR generated EcoRI-SmaI cDNA fragment was used for purification of the glutathione S-transferase (GST)-fusion protein. The EcoRI-SmaI cDNA fragment was generated using primer 5AD and primer 2y14Sma-3 containing a SmaI site in the 3'end.

The GST pull-down experiment was performed as described before. ^{35}S-labeled RSI protein was detected in the SDS gel by autoradiography (Fig. 11).

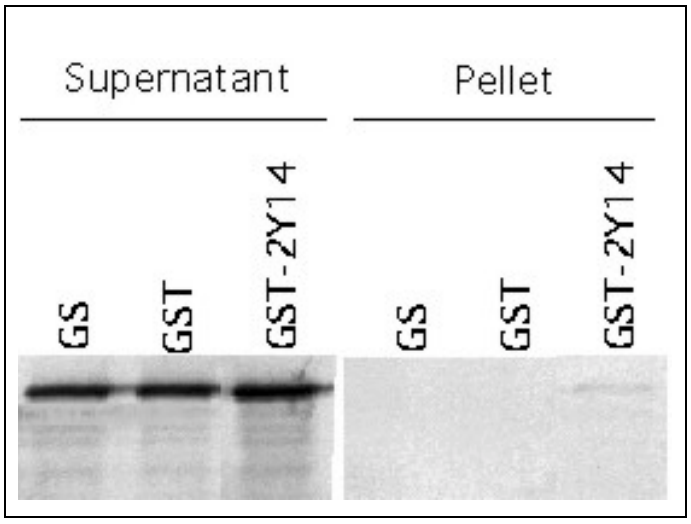

Fig.11. *In-vitro* GST-2Y14 fusion protein pull-down assay with ^{35}S-labeled RSI. The empty matrix (GS), control GST and GST-2Y14 (10 µg of each) bound to glutathione-agarose were incubated with 2.5 µl of *in vitro* translated ^{35}S-labeled RSI using TNT® Coupled Reticulocyte Lysate Systems. The separation and detection of protein followed the way of clone Y6.
Empty matrix (GS): glutathione-agarose beads, control GST: glutathione S-transferase (GST) protein, and GST-2Y14: GST-2Y14 fusion protein.

3.3.5.2 Summary of clone 2Y14 properties

2Y14 displays homology to centromere protein homologs from *Arabidopsis* and to a Rho-associated coiled-coil containing protein kinase, p160 ROCK-2, from *Mus musculus*. 2Y14 interacts with RSI in the yeast two-hybrid system and also *in vitro*, as judged from a GST pull-down experiment. The interaction of 2Y14/RSI was extremely weaker compare to other interaction.

3.3.6 Clone 2Y16 (AmGRAS)

A BLAST search has revealed that 2Y16 contains highly conserved domains like GAI, RGA, and SCR which belong to the GRAS transcription factor family of *Arabidopsis*. This clone was chosen and further characterized. 2Y16 was renamed to AmGRAS (*Antirrhinum majus* GRAS protein). The alignment of AmGRAS with other GRAS family proteins is shown in Fig. 15. Several conserved domains are detectable which will be explained in detail later.

3.3.6.1 *In-vitro* GST–AmGRAS fusion protein pull-down assay

To confirm the protein-protein interaction by an independent method, an *in vitro* GST pull-down assay was carried out. A PCR generated BamHI-EcoRV cDNA fragment containing the AmGRAS ORF was used for the isolation and purification of the glutathione S-transferase (GST)-fusion protein. The BamHI-EcoRV cDNA fragment was generated using primer 2y16BamHI-5 (introducing a BamHI site in the 5'end) and primer 2y16EcoRV-3 primer (introducing an EcoRV site in the 3'end).

The opposite combination with RSI as GST-fusion protein was also tested to be sure the AmGRAS/RSI interaction in a more confirmative way. The EcoRI-SalI cDNA fragment of RSI from the pBluescript® was cloned into the pGEX-5X-1 and used for the purification of GST-

RSI fusion protein.

A cDNA fragment of RSI (obtained from Dr. M. Roccaro) served as a template to synthesise [^{35}S] methionine-labeled proteins in a TNT® Coupled Reticulocyte Lysate Systems (Promega). A PCR generated NcoI-PstI cDNA fragment of *AmGRAS* was cloned in pGBKT7 and served to synthesize [^{35}S] methionine-labeled protein. ^{35}S-labeled RSI or AmGRAS proteins were detected as matrix-bound proteins by autoradiography (Fig.12 and Fig. 13).

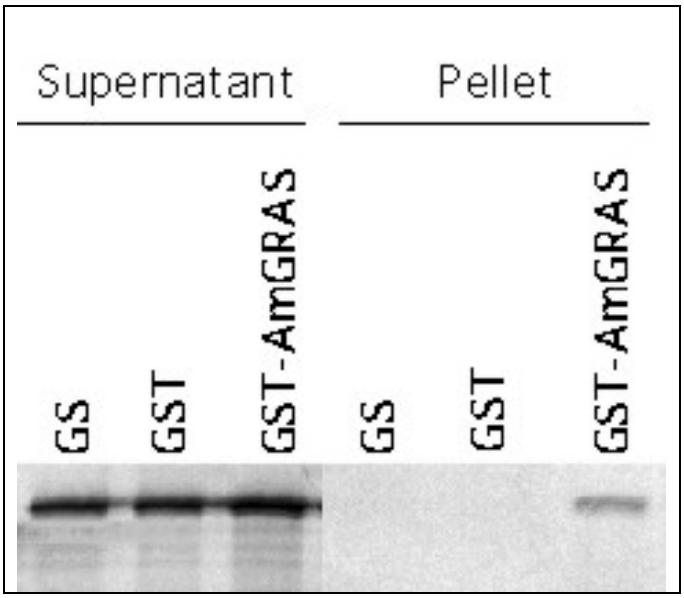

Fig.12. *In-vitro* GST-AmGRAS fusion protein pull-down assay with ^{35}S-labeled RSI. The empty matrix (GS), control GST and GST-AmGRAS (10 µg of each) bound to glutathione-agarose were incubated with 2.5 µl of *in vitro* translated ^{35}S-labeled RSI using TNT® Coupled Reticulocyte Lysate Systems. The separation and detection of protein followed that of clone Y6.
Empty matrix (GS): glutathione-agarose beads, control GST: glutathione S-transferase (GST) protein, and GST- AmGRAS: GST-AmGRAS fusion protein.

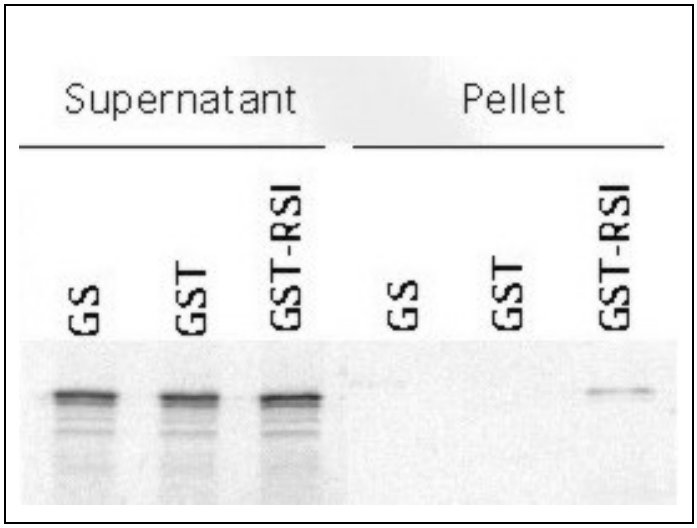

Fig.13. *In-vitro* GST-RSI fusion protein pull-down assay with ^{35}S-labeled AmGRAS. The empty matrix (GS), control GST and GST-RSI (10 µg of each) bound to glutathione-agarose were incubated with 2.5 µl of *in vitro* translated ^{35}S-labeled AmGRAS using TNT® Coupled Reticulocyte Lysate Systems. The separation and detection of protein followed the way of clone Y6.
Empty matrix (GS): glutathione-agarose beads, control GST: glutathione S-transferase (GST) protein, and GST-RSI: GST-RSI fusion protein.

3.3.6.2 Summary of clone 2Y16 features

2Y16 displays high sequence homology with GAI, RGA, and SCR which belong to the GRAS transcription factor family of *Arabidopsis*. 2Y16 interacts with RSI in the yeast two-hybrid system and also *in vitro*, as judged from a GST pull-down experiment.

3.3.7 Clone 2Y19

Clone 2Y19 was isolated as a 800 bp fragment. It displayed sequence similarity with a putative N-methyl transferase and a hypothetical protein from *Arabidopsis*. Interestingly, this protein showed high β-galactosidase activity in yeast and strong interaction in the *in vitro* GST pull-down assay. It does not contain a transcription activation domain.

3.3.7.1 *In-vitro* GST–2Y19 fusion protein pull-down assay

A PCR genetated EcoRI-XhoI cDNA fragment containing the 2Y19 ORF was used for purification of the GST-fusion protein. The EcoRI-XhoI cDNA fragment was generated using primer 5AD and primer 2y19Xho-3 primer (introducing a *XhoI* site in the 3'end).

The GST pull-down experiment was performed as described before. ^{35}S-labeled RSI protein was detected by autoradiography as described before (Fig. 14).

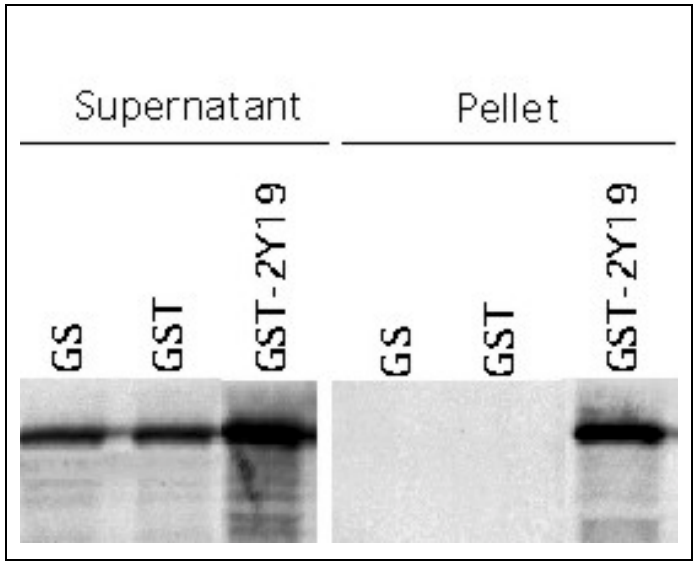

Fig.14. *In-vitro* GST-2Y19 fusion protein pull-down assay with ^{35}S-labeled RSI. The empty matrix, control GST and GST-2Y19 (10 µg of each) bound to glutathione-agarose were incubated with 2.5 µl of *in vitro* translated ^{35}S-labeled RSI using TNT® Coupled Reticulocyte Lysate Systems. The separation and detection of protein followed the way of clone Y6.
Empty matrix (GS): glutathione-agarose beads, control GST: glutathione S-transferase (GST) protein, and GST-2Y19: GST-2Y19 fusion protein.

3.4 AmGRAS is a new member of the GRAS transcription factor family

Most of the putative interaction partners did not show high sequence similarity to database entries or, if they did, the function of the proteins is unknown. 2Y16 is the exception, because it shows high sequence similarity to members of the GRAS (formerly called VHIID) transcription factor family, such as RGA and GAI from *Arabidopsis* (Silverstone *et al*., 1998, Peng J *et al*., 1997).

The predicted AmGRAS protein contains 528 amino acids with an estimated molecular mass of approximately 58 KDa. AmGRAS shares 70% sequence identity with RGA and 69% sequence identity with GAI, and belongs to the plant specific GRAS (for GAI, RGA, SCARECROW) family of regulatory proteins (Pysh *et al*., 1999). Other members of this family

include Rht-D1 from wheat (Harberd *et al*., 1989), D8 from maize (Winkler *et al*., 1994), Ls, a protein from tomato that is required for formation of axillary branches and which shows high sequence similarity with other GRAS family members in the carboxy–terminal region (Schumacher *et al*., 1999) and PAT1, which is involved in phytochrome A signal transduction (Bolle *et al*., 2000). Both D8 and Rht-D1, are functional orthologues of the *Arabidopsis* GAI and RGA factors, which are involved in the gibberellin signaling pathway.

The first member of the GRAS family to be identified was SCARECROW (SCR). The SCR gene regulates asymmetric cell division of cortex/endodermis initial cells during root development (Di Laurenzio *et al*., 1996). The sequence similarity of SCR with other GRAS family members such as Ls (Schumacher *et al*., 1999) is limited to the carboxy–terminal region.

Up to now, more than 38 GRAS family members have been identified in *Arabidopsis*. All GRAS family proteins contain the highly conserved DELLA domain, two large leucine heptad regions, nuclear localization signals (NLS), a VHIID domain (for the conserved Val-His-Ile-Ile-Asp), a LXXLL domain, a RVER domain, a SH2-like domain, and a SAW domain (Fig. 15). The features of these domains are described in the following.

DELLA domain:

AmGRAS, RGA, GAI and several other members have a unique conserved region near the amino terminus called DELLA. This region seems to be involved in modulating GA response, because the gain-of-function *gai-1* allele contains a deletion of 17-amino acids in the amino terminal DELLA domain resulting in a mutant protein which causes a reduction in GA responses.

Leucine heptad repeats:

Two leucine heptad repeats are found before and after the VHIID domain in the GRAS family proteins. The presense of leucine heptad repeats is a strong indication of protein-protein interaction as demonstrated for other proteins (Hurst, 1994), and this was proven again by the interaction of AmGRAS with RSI.

VHIID domain:

This domain is common in all members of the GRAS family, although it is not absolutely conserved. The VHIID domain is more accurately described as (V/I)H(V/I)(V/I)D, where V is valine, I is isoleucine, H is histidine, and D is aspartic acid.

LXXLL domain:

This motif fits the consensus amino acid sequence LXXLL, where L is leucine, and X is any amino acid. It was demonstrated that it mediates the binding of steroid receptor co-activator complexes to nuclear receptors (Heery *et al*., 1997).

SH2-like domain:

The putative SH2 (Src homology 2) domain binds certain phosphotyrosine containing proteins and is thought to be involved in protein-protein interaction in signal transduction. The SH2 domain has been identified in Rht, D8, GAI, and RGA (Peng *et al*., 1999). This domain is present in a family of transcription factors called STATs (Signal Transducers and Activators of Transcription) in animals (Darnell *et al*., 1997). The function of this domain is to mediate the binding of STATs to various receptor tyrosine kinases. The STATs are then activated by the recpetor kinase , and translocated from the cytoplasm to the nucleus.

A typical SH2 domain is a peptide stretch of 100 amino acids containing an invariant

arginine (R) that recognizes the phosphate group of phosphotyrosine.

RVER domain:

Conserved set of amino acids located at the C-terminus, of which no function is known until now.

SAW domain:

This domain is also conserved set of amino acids (Serine, Alanine, and Tryptophan) located at the C-terminus. No function is known until now.

A sequence comparison of AmGRAS with GRAS family proteins is shown in Fig. 15.

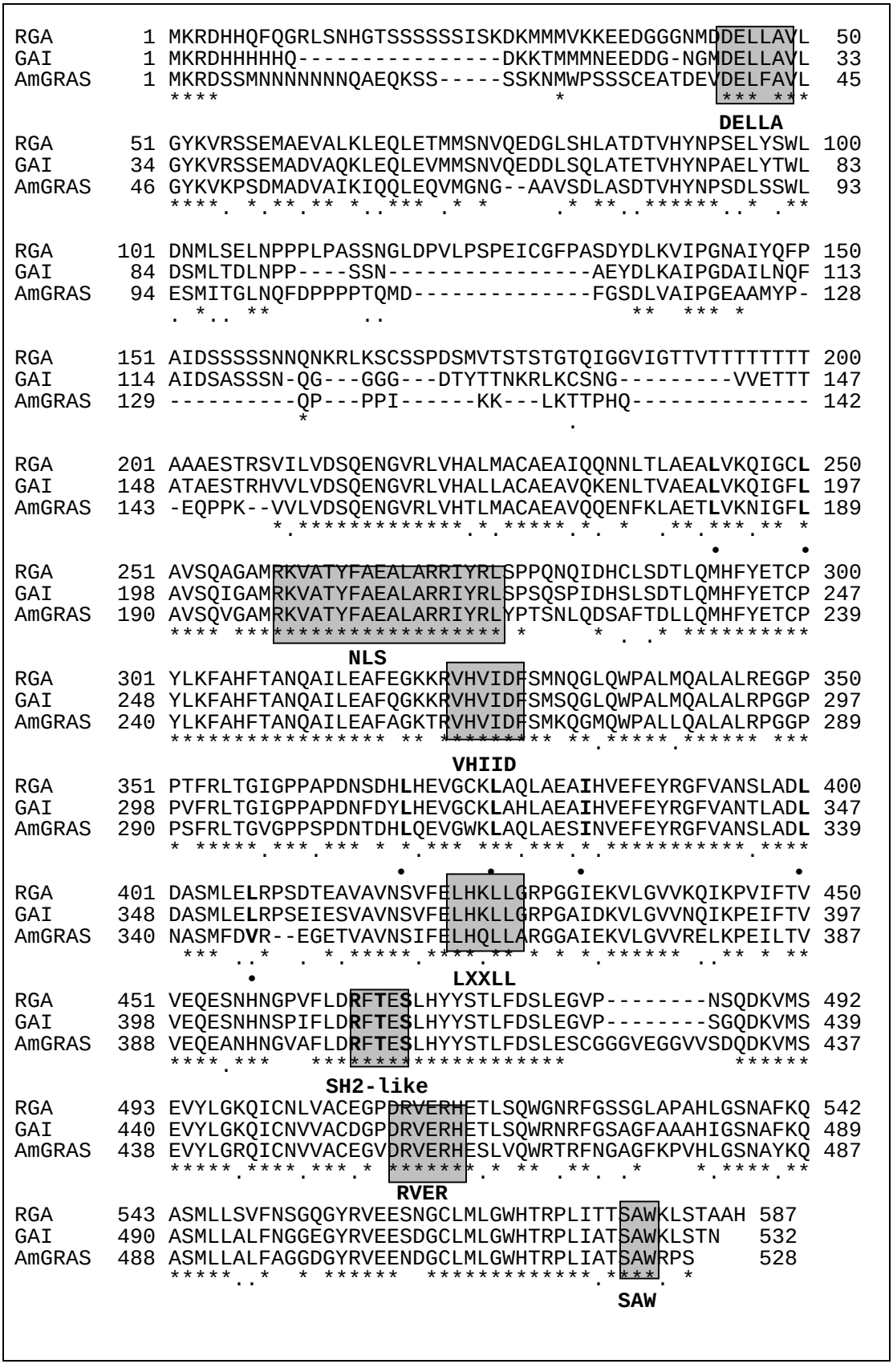
DELLA
NLS
VHIID
LXXLL
SH2-like
RVER
SAW

Fig. 15. Amino acid sequence comparison of the AmGRAS protein with other members of the GRAS family.

The AmGRAS amino acid sequence is compared with RGA (Silverstone et al., 1997a) and GAI (Peng et al., 1997). Identical residues conserved between AmGRAS and other GRAS family member are indicated with a star (*) in the bottom line, and similar residues are indicated with a dot (.) in the bottom line. Gaps introduced to improve the alignment are indicated by blanks, and sequence truncations by dashes. The acidic DELLA domain, NLS, VHIID, LXXLL, SH2, RVER and SAW domains are underlined. The leucine heptad repeat regions are marked with a triangle (•), and each leucine is marked in bold. In SH2-like domain, invariant arginene (R) and other strongly conserved residues (T and S) are bold.

3. 5 Evolutionary relationships of *AmGRAS* with other GRAS family genes

To determine the evolutionary relationship of AmGRAS with the other members of the GRAS family, a phylogenetic tree was constructed using the MacVector program. The tree was generated using amino acid sequences of GRAS family proteins from different species.

RGA and GAI are closely related. They share 73 % amino acid sequence identity and 7 % amino acid sequence similarity. *AmGRAS* has a higher nucleic acid sequence similarity with *RGA* (70%) than with *GAI* (69%). However, at the protein level, AmGRAS is more related to GAI than to RGA (Fig. 16). AmGRAS shares 66% identity and 11% similarity with GAI, whereas it has 59% identity and 9% similarity with RGA at the amino acid sequence level.

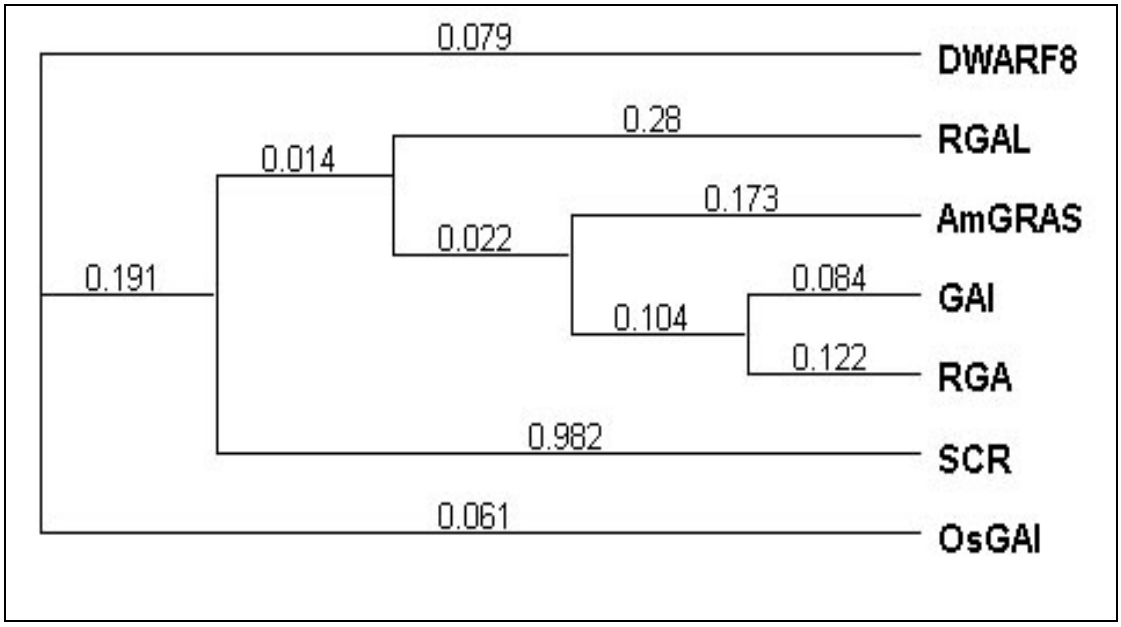

Fig. 16. Phylogenetic tree of AmGRAS and other GRAS family proteins.
The tree was generated using the neighbor joining method of the MacVector program. Gap penalty was 10 and extended gap penalty was 0.1. DWARF8 (AAL10303) is from maize, OsGAI(BAA90749) is from rice, and RGA(CAA72177), GAI(CAA75492), RGAL(CAA12242), and SCR(NM_124189) are from *Arabidopsis*.

3.6 Isolation and characterization of the *AmGRAS* gene

In order to obtain information about the structure of the AmGRAS gene, and especially about the promoter, an attempt was made to isolate genomic clones from a λEMBL4 library of *A.majus*. The library was screened with a radioactively labeled *AmGRAS* cDNA. Half a million recombinants were screened, 18 positive candidates were isolated. Two EMBL3 vector primers (EL1 and ER2), flanking the insert, and a gene specific primer (BGA5) were used for PCR to obtain information about the orientation of the insert and about the size. From one strongly hybridizing clone a 3.7kb long fragment was amplified by PCR with BGA5 and ER2. The gene sequence of *AmGRAS*, and of the promoter, could be obtained by 'primer walking' sequencing.

Two additional primers (BGA3 and BGA4) were used to obtain 1.5 kb sequence upstream from the translation start site.

DNA sequence analysis of the genomic DNA revealed that the AmGRAS has an interrupted 1585 bp ORF with no introns.

A putative TATA-box is indicated in Fig. 17. It was identified by using a promoter search program (http://www.fruitfly.org/cgi.bin/seq_tools/promoter). Several other transcription factor binding sites are predicted by the TFSEARCH search program (http://www.cbrc.jp/research/db/ TFSEARCH.html). The putative core motifs are marked in Fig. 17. in bold and by underlining.

Four core motifs for a petal epidermis-specific MYB transcription factor, similar to that for MYB.ph3 from *Petunia hybrida* binding site, CNGTT(A/G) or AGTTAGTTA (Solano *et al*., 1995) and one core motif for SBF-1, a GTTA motif closely related to the GT-1 binding site, GGTTAA(A/T)(A/T)(A/T), (Lawton *et al*., 1991) were found. MYB.ph3 is localized in the epidermal cell layer of petals, where flavonoid biosynthetic genes are actively expressed. SBF-1 specifically interacts with regulatory sequences in the promoter of the bean defense gene CHS15, which encodes the flavonoid biosynthetic enzyme chalcone synthase. GT-1 is involved in the light-dependent expression of the ribulose bisphosphate carboxylase small subunit gene in green tissue.

TTTTTTTTTTTTTTTTATAATTGTCCCTTACAAGAACCTTTGGACATTGC -1507

TCATCTGTAAAAAAATGTATTCTAGTCATTTATTGACCAAAAATGTACAT -1457

CTGAGATTCCGTTACCTTTGGAAAAATATTAATTGTCAGAGAATAGTTTA -1407

TTGGAACTCTGAAAAGTTAACTTCCATCTCACTAAAAGCAAAGTTACAAG -1357

TAATCCCTTTTTCTTTGTTTGAGGATTTTGTAAACTGCATCAGTTGACTA -1307

AGCATGCAGGTTACGAAGTTAACCCAAATACAAGCAATTTGCACTTTAAG -1257

AATTTATCATCAATTTCTTCCTTCCACTTCACCAGTAAAAAAGCATTCCA -1207

GTAACAAAAATAACTGTATTTATGGTGGTAAAAGAAAAAAGAAACGATAA -1157

CTTTTAATTAGTAAAAGAGTGCCGACCTGTTAAATGAACCGTACTCATTT -1107

AAAGTTGGACTAGGTAGTACGCTTAGTTATATAGTACTAATTACTGAAAC -1057

CATACTGCCAATTGTGTAC**TAAAGTTAGATAA**TATATTAGGCAAATTTGC -1007

AAGCATTTTCCATTTGTGGAAGTCTAAACTATCTTTAAAGGCAAC**TTAAC** -957

TTACTTTTTTTTATGAAA**ACCCACCTT**AGTTTCCATATAAGCTAAAATAT -907

TTTGTTGCATAAAAATTTCGGTACCCATAAATTTAGCAAGGTGTCATTTT -857

AAACGATTTCTAACAATTAAACTGAAATTGTT***TTCTGTTTAATATT***AAAG -807

TTTAAATACCACGCACTAATATTTTTTTTTTAAATTTTAAATTTTCCATT -757

TATTTGTCCAGAATGTAGAAATAAAAGTACCTTTTGCAAATGACGTTCCA -707

GATGATAGCTGCATGAGCTCAATTTACTTTTAATTTTCTAATAAACCAAT -657

ATTAAACGCCTCTTATTTTTAACACAAAGCTAAGATAACTGGTGATTTTT -607

CGCAACAAAGAATGTCAATATGATGATAATCTAAAATATTTA**AGGAGTAA** -557

GTTAAGAAGTGGCA**TATATATA**TTTGTAGCATCTGAAAAAATTCTAAAAA -507

ATTGAACATGAACATTATACATCACATTGACACAGGTAAATAAATAGTCT -457

CGAGAACTCTCGTATGTTTATCATCAGTAAAAGATTATGAGTCACAGAGT -407

GTAACAAATTATTACCGCTTACTACAATAATAAGCATCTGGTGGAAAAAA -357

ATAAAATAAAAAAGCATAAAAAAGTGGATATTATTTTTTGTATGTAAAAT -307

TGATCTGCTGTTGCTGTTGCTGGTGCTGGTGCAGCAGCAATAATATTGAT -257

AAGTGTGTATTGAAGGTGGCGCCCCCACCTTCACGAGCCTTTCCTTTTCT -207

CTCTCTATTCTATTATTATACTATACTCCCTCCAATCACATCACTTTTTC -157

TCTCTCCTCTCCCCAAAACACACACACACACACACACACACACACACTTT -107

CTTCAATCCCCCAATGCTCTACTTCTTCTTCCTCACAGAAACAAAAGAAA -57

ATAAAAAATGAAAAGGGATAGCAGTATGAACAACAACAACAACAACAACC 43

M K R D S S M N N N N N N N

AAGCTGAACAGAAGAGCAGTTCCTCAAAGAACATGTGGCCCTCCTCCTCC 93

Q A E Q K S S S S K N M W P S S S

TGCGAGGCAACCGACGAGGTGGACGAGCTCTTCGCCGTACTAGGTTACAA 143

C E A T D E V D E L F A V L G Y K

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GGTCAAACCCTCCGACATGGCCGACGTGGCAATCAAGATCCAGCAGCTCG  193
  V  K  P  S  D  M  A  D  V  A  I  K  I  Q  Q  L
AGCAAGTCATGGGCAACGGCGCCGCCGTCTCGGATCTCGCATCCGACACC  243
E  Q  V  M  G  N  G  A  A  V  S  D  L  A  S  D  T
GTCCACTACAACCCCTCCGATCTCTCCTCCTGGCTCGAGTCCATGATCAC  293
 V  H  Y  N  P  S  D  L  S  S  W  L  E  S  M  I  T
CGGCCTCAACCAATTCGACCCGCCTCCCCCGACCCAGATGGATTTCGGGT  343
  G  L  N  Q  F  D  P  P  P  P  T  Q  M  D  F  G
CGGATCTCGTCGCCATACCCGGTGAGGCCGCCATGTACCCTCAACCCCCA  393
S  D  L  V  A  I  P  G  E  A  A  M  Y  P  Q  P  P
CCCATCAAAAAACTCAAAACCACACCGCATCAAGAGCAGCCGCCTAAAGT  443
 P  I  K  K  L  K  T  T  P  H  Q  E  Q  P  P  K  V
GGTGCTTGTTGACTCACAGGAGAACGGCGTGAGGCTGGTGCACACTCTAA  493
  V  L  V  D  S  Q  E  N  G  V  R  L  V  H  T  L
TGGCTTGTGCCGAGGCCGTACAGCAGGAGAATTTCAAACTAGCGGAGACT  543
M  A  C  A  E  A  V  Q  Q  E  N  F  K  L  A  E  T
CTTGTCAAGAACATTGGGTTTTTAGCCGTTTCTCAAGTGGGTGCTATGCG  593
 L  V  K  N  I  G  F  L  A  V  S  Q  V  G  A  M  R
TAAGGTCGCTACTTATTTTGCTGAGGCTTTGGCCAGGAGAATCTACAGGT  643
  K  V  A  T  Y  F  A  E  A  L  A  R  R  I  Y  R
TGTATCCCACATCGAATCTGCAAGACTCCGCCTTTACGGATTTGCTGCAA  693
L  Y  P  T  S  N  L  Q  D  S  A  F  T  D  L  L  Q
ATGCATTTCTACGAGACTTGTCCGTACCTCAAGTTCGCGCATTTCACGGC  743
 M  H  F  Y  E  T  C  P  Y  L  K  F  A  H  F  T  A
GAATCAAGCGATTCTCGAGGCTTTTGCGGGTAAGACGAGAGTACACGTGA  793
  N  Q  A  I  L  E  A  F  A  G  K  T  R  V  H  V
TTGATTTTAGTATGAAGCAGGGTATGCAGTGGCCTGCTCTGTTACAGGCT  843
I  D  F  S  M  K  Q  G  M  Q  W  P  A  L  L  Q  A
TTGGCCTTGCGTCCCGGGGGCCCGCCCAGCTTTCGATTAACCGGGGTCGG  893
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GCCTCCGTCGCCCGATAACACCGATCATTTGCAGGAGGTTGGATGGAAAT  943
  P  P  S  P  D  N  T  D  H  L  Q  E  V  G  W  K
TGGCGCAATTGGCTGAATCGATAAACGTCGAGTTTGAGTACAGAGGGTTT  993
L  A  Q  L  A  E  S  I  N  V  E  F  E  Y  R  G  F
GTGGCGAATTCGTTGGCTGATTTGAATGCGTCGATGTTTGATGTGCGTGA 1043
 V  A  N  S  L  A  D  L  N  A  S  M  F  D  V  R  E
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GGGTGAGACTGTTGCGGTGAATTCGATATTCGAGCTGCATCAGTTGTTGG 1093
  G  E  T  V  A  V  N  S  I  F  E  L  H  Q  L  L
CGAGGGGTGGAGCAATTGAGAAGGTGTTGGGTGTGGTTAGAGAGTTGAAG 1143
A  R  G  G  A  I  E  K  V  L  G  V  V  R  E  L  K
CCTGAGATTTTGACTGTTGTTGAGCAAGAAGCTAATCACAATGGGGTTGC 1193
 P  E  I  L  T  V  V  E  Q  E  A  N  H  N  G  V  A
GTTCTTGGACAGGTTTACGGAGTCGTTGCACTATTATTCGACCCTTTTCG 1243
  F  L  D  R  F  T  E  S  L  H  Y  Y  S  T  L  F
ACTCGTTGGAGAGCTGCGGTGGCGGTGTCGAAGGGGGCGTGGTGAGCGAT 1293
D  S  L  E  S  C  G  G  G  V  E  G  G  V  V  S  D
CAAGATAAGGTGATGAGTGAGGTTTATTTGGGTCGACAAATCTGTAATGT 1343
 Q  D  K  V  M  S  E  V  Y  L  G  R  Q  I  C  N  V
GGTGGCGTGTGAGGGAGTGGACCGAGTCGAGAGGCACGAGAGCTTGGTTC 1393
  V  A  C  E  G  V  D  R  V  E  R  H  E  S  L  V
AGTGGCGAACCCGGTTCAATGGTGCCGGTTTTAAGCCGGTTCATTTGGGT 1443
Q  W  R  T  R  F  N  G  A  G  F  K  P  V  H  L  G
TCCAATGCTTATAAGCAAGCTAGTATGCTGTTGGCTTTGTTTGCAGGTGG 1493
 S  N  A  Y  K  Q  A  S  M  L  L  A  L  F  A  G  G
TGATGGGTATAGAGTTGAGGAGAACGATGGGTGTTTGATGTTGGGATGGC 1543
  D  G  Y  R  V  E  E  N  D  G  C  L  M  L  G  W
ACACGAGGCCATTGATTGCTACATCGGCTTGGAGGCCGAGTTGACTCACT 1593
H  T  R  P  L  I  A  T  S  A  W  R  P  S  *
AACTCACTGAGTTGGTATTCTTTTGGCTGAGTTTAGTGGGTGTTTTGCTG 1643
AGCTGTTTGGTTGTTAAGGGTGAGACCCCACCACTCGTTTAATGGTGTCA 1693
CTCTCAAGTTCGATCTTTTGATGCTGCTCTTTCTTTTCTCGTAGCTTTTT 1743
GCCCTATCTTTTTTTAGTTTTTAATTTAAATTTAAAGTTTATTTGGTTGA 1793
ATCTCTTGTAATTCTCGGAGGGGGATCTGAAGTGGATGTGTATTTATTAT 1843
ATTTATATATATAGCTCTTTTTTCTTTAGCAAAATAAAAGGGTGTGGAGC 1893
TTTTGGGATATTTGAGTATGTGTTTGATGGCAAAAA               1929
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Fig. 17. Genomic structure of the *AmGRAS* gene.

The deduced amino acid sequence of *AmGRAS* is shown below the nucleotide sequence. The probable TATA box is in bold. Four times possible binding sites for a petal epidermis-specific MYB transcription factor like MYB.ph3 from *petunia hybrida*, are bolded and underlined. A SBF-1, closely related to GT-1, binding site is indicated in italics and bold.

3.7 *AmGRAS* is a single-copy gene

RGA and GAI, members of the GRAS family of transcription factors in *Arabidopsis* are single copy genes (Silverstone AL *et al*., 1998, Peng J *et al*., 1997). To test whether this is also true for AmGRAS of *Antirrhinum*, Southern hybridization was carried out using various restriction enzymes for digestion of genomic DNA. The digested DNAs were separated on a 0.8% agarose gel and blotted on nylon membrane. The filter was hybridized with α ^{32}P-labeled *AmGRAS* cDNA probe at high stringency (68°C) and washed at the same temperature and exposed to an X-ray filter. The result is shown in Fig. 18. Only one band is visible in each lane, suggesting that *AmGRAS* is a single copy gene.

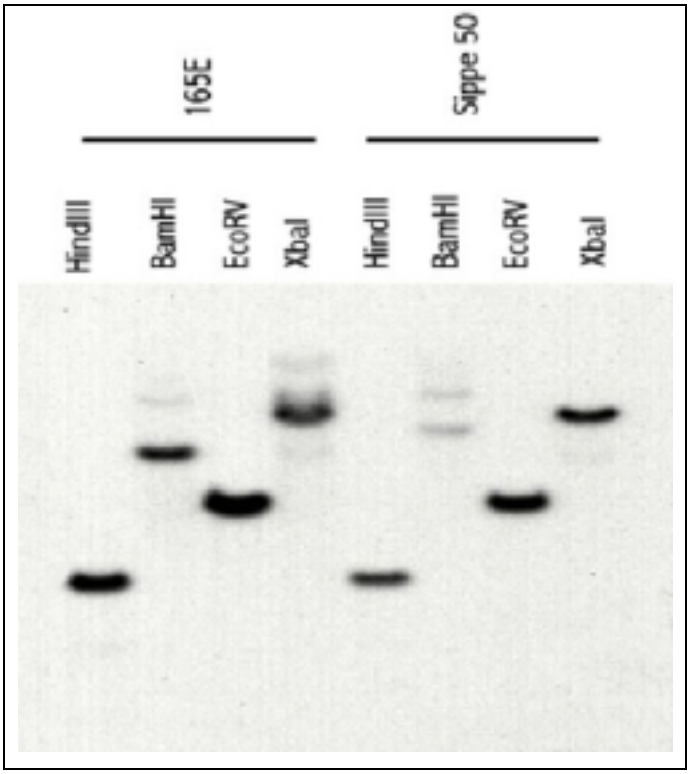

Fig.18. Genomic Southern blot analysis of the *AmGRAS* gene at high stringency. *Antirrhinum* genomic DNA (3 µg per lane) from two different wild type lines (165E and Sippe50) was digested with various enzymes indicated above the lanes, size separated on a 0.8% agarose gel and capillary blotted onto a nylon membrane. The blot was probed with α-^{32}P dCTP labeled *AmGRAS* cDNA in 3×SSPE and 0.1%SDS at 68°C. The blot was washed with 2×SSPE, 0.1%SDS and 1×SSPE, 0.1%SDS, respectively, at 68°C for ca. 1-2hr and exposed to X-ray film at -70°C for 5 days.

To get insight whether *AmGRAS* is also a member of a gene family, like *GAI* and *RGA* of *Arabidopsis*, Southern hybridization at lower temperatures, at 60°C and 58°C, was performed (Fig. 19). The blot previously used for genomic Southern hybridization of *AmGRAS* was stripped to remove the probe and re-used for hybridization at lower temperatures with *AmGRAS* as probe.

The hybridizations at 60°C and at 58°C show one to three additional weaker signals which indicates the existence of a GRAS-like family also in *Antirrhinum*.

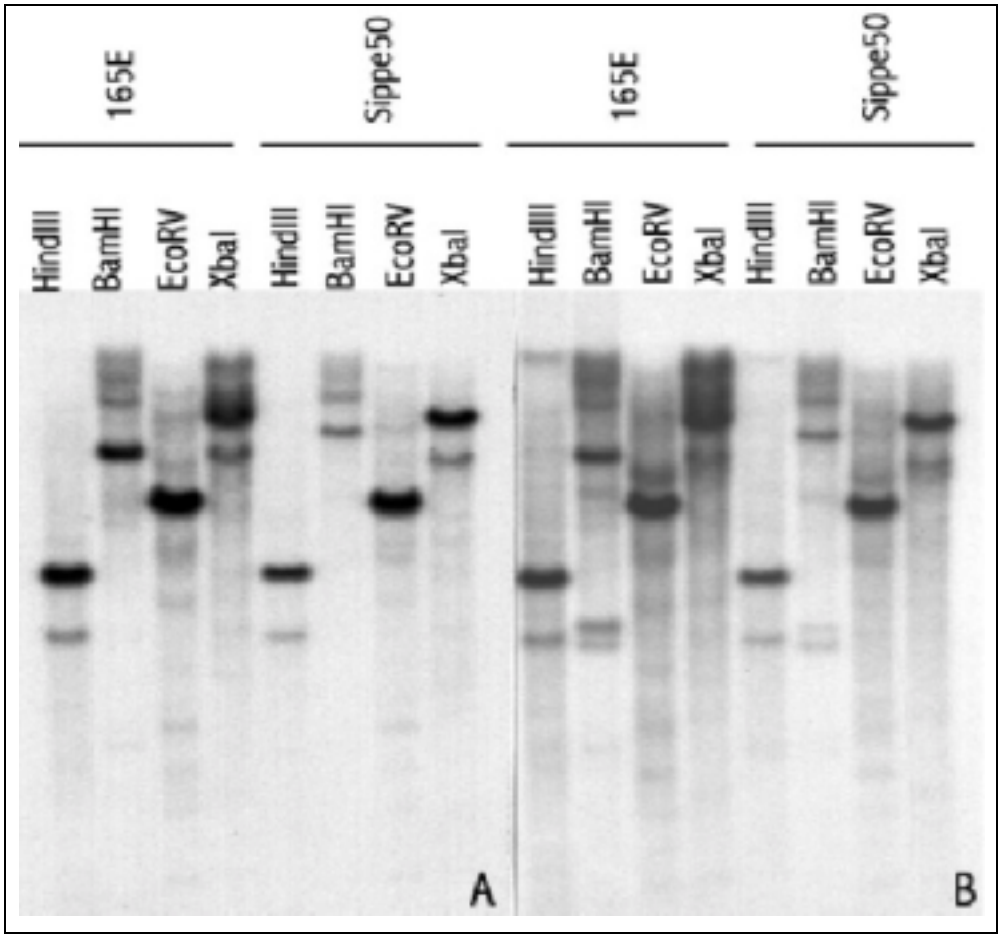

Fig. 19. Genomic Southern blot analysis of the *AmGRAS* gene at low stringency condition. *Antirrhinum* genomic DNA (3 µg per lane) from two different wild type (165E and Sippe 50) lines was digested with various enzymes indicated above the lanes, size separated on a 0.8% agarose gel and capillary blotted onto a nylon membrane. The blot was probed with α-^{32}P dCTP labeled *AmGRAS* cDNA in 3×SSPE and 0.1% SDS at 60°C (A), and at 58°C (B). The blot was washed with 2×SSPE, 0.1% SDS and 1× SSPE, 0.1% SDS, respectively, at 60°C (A), and 58°C (B) for ca. 1-2hr and exposed to X-ray film at -70°C for 5 days.

3.8 Spatial and temporal expression pattern of *AmGRAS*

To gain insight into the possible function of *AmGRAS* in plant organs and to obtain information about its expression, the spatial and temporal expression pattern of *AmGRAS* was analyzed.

The expression pattern of *AmGRAS* in several plant organs was investigated by Northern hybridization with mRNA isolated from different flower organs and leaves.

As Fig. 20 shows, *AmGRAS* is expressed in all plant tissues tested, with the corresponding transcript length of 2.4 kb. The transcript of *AmGRAS* was also detected in roots by RT-PCR.

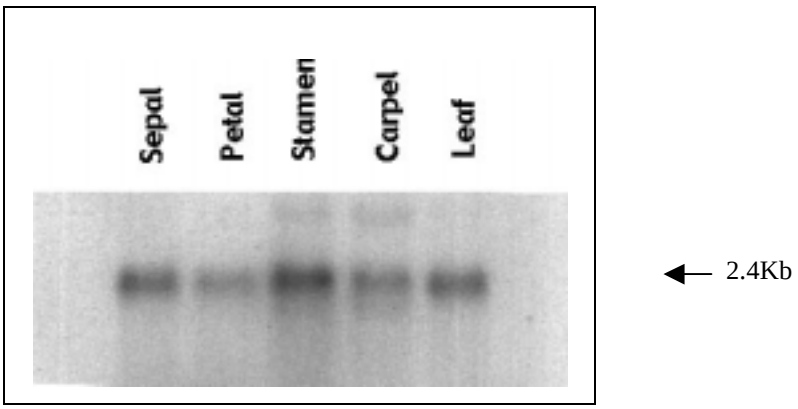

Fig. 20. Expression pattern of the AmGRAS gene.
Each lane contains 2 μg of mRNA isolated from different tissues, as indicated above the lanes. The mRNAs were size separated on a 0.8% agarose gel and capillary blotted onto a nylon membrane. The blot was probed with α-^{32}P dCTP labeled *AmGRAS* cDNA in 50% deionized formamide, 5× SSPE, 10× Denhardt mix, 0.5% SDS and 100 μg/ml of salmon sperm DNA. The blot was washed with 2× SSPE, 0.1% SDS at 25°C /1× SSPE, 0.1% SDS at 40°C, and exposed to X-ray film at -70°C for 5 days.

In situ hybridization was used to determine the spatial mRNA expression pattern of *AmGRAS* more precisely. To avoid cross hybridization with other GRAS family members, probes derived from the least conserved regions have been used, 5' AmGRAS and 3' AmGRAS

(Fig. 21). The 5' AmGRAS probe is located between the DELLA domain and the NLS domain, and the 3' AmGRAS probe corresponds to the 3'UTR region (Fig. 21). The BamHI/PstI fragment of 5' AmGRAS was obtained by PCR amplification using the primers BamHIGAI and PstBGA5, and the 3' AmGRAS fragment by PCR amplification using the primers 2Y16BamHI-end and 2Y16PstIend. The fragments were cloned into the pBluescript® SK vector. The antisense probe was generated using T7 RNA polymerase.

Sectioning and *in situ* hybridization was carried out as described in Materials and Methods.

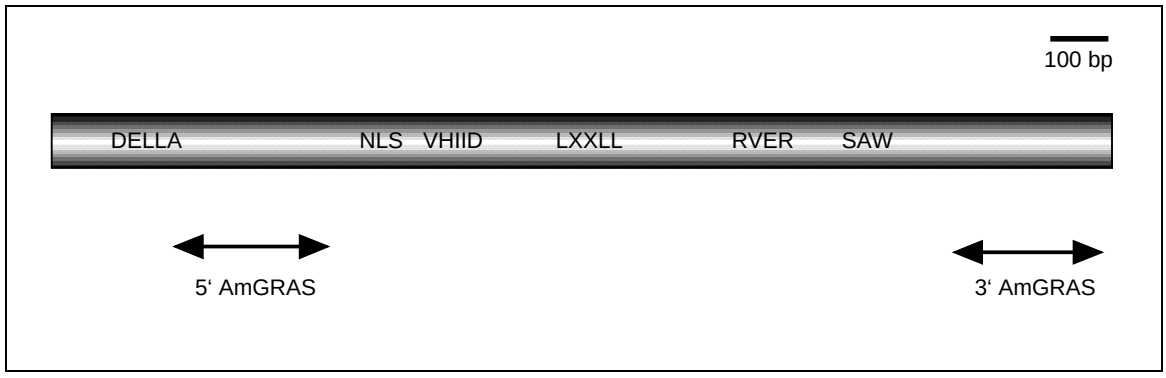

Fig. 21. Schematic representation of the *AmGRAS* cDNA and the regions from which the probes for *in situ* hybridization were derived.

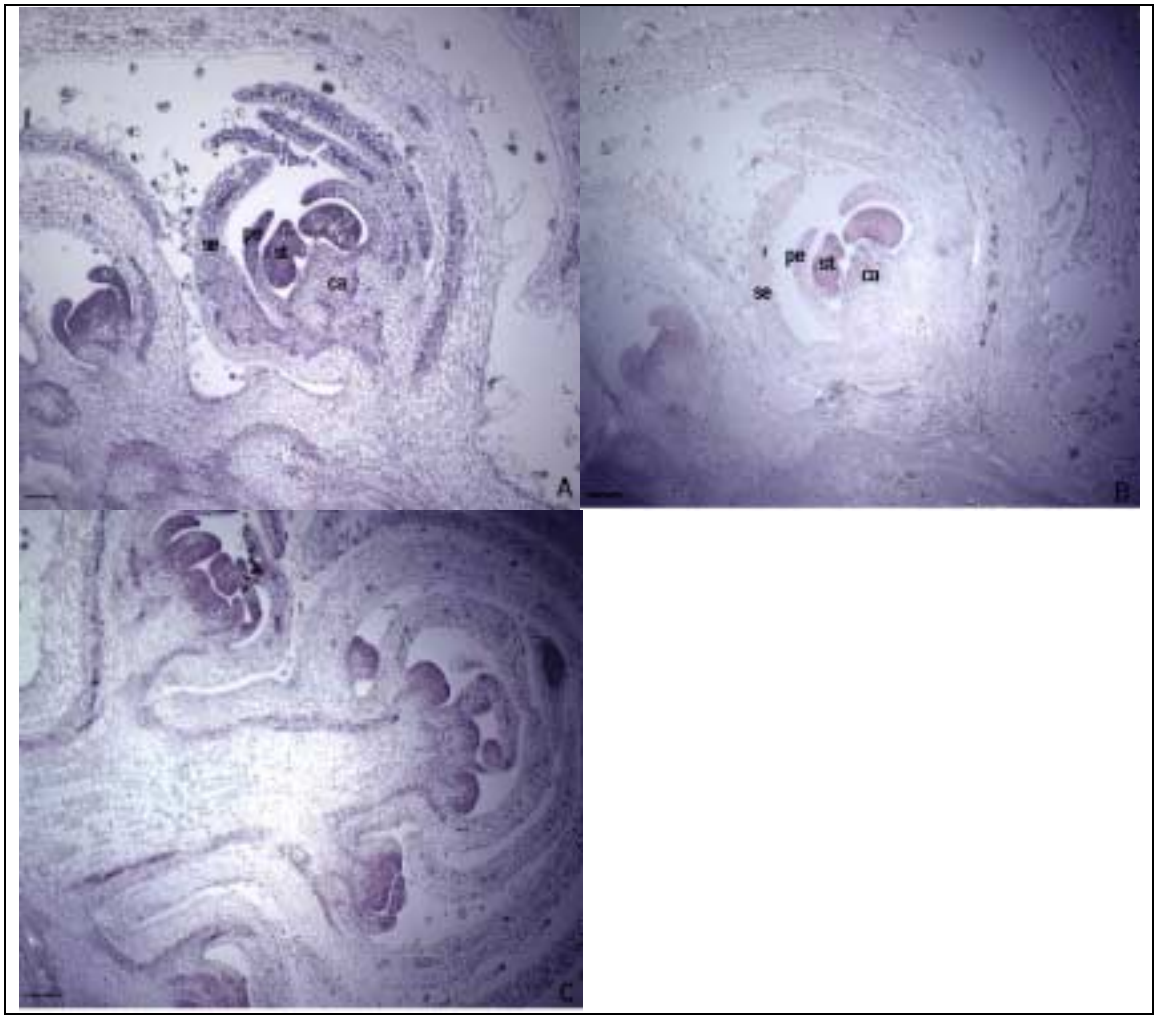

Fig. 22. *In situ* hybridization with *AmGRAS*. Longitudinal sections of young floral buds were hybridized with either an antisense 5'*AmGRAS* riboprobe (A and C) or a sense 5'*AmGRAS* riboprobe (B).
Antisense and sense riboprobes were labeled with digoxigenin-11-rUTP using the Boehringer nucleic acid labelling kit. DNA templates were either cDNAs subcloned into pBluescript SK and prepared by PCR using T3/T7 primers. T7 RNA polymerase was used to synthesize antisense DIG-RNA.
se: sepal, pe: petal, st: stamen, ov: ovule. Scale bars in A, B and C are 100 μm.

As shown in Fig. 22, expression of *AmGRAS* in early stages was dectected in several different tissues, such as the inflorescence apical meristems, floral meristem, and flower organ primordia. Therefore, AmGRAS expression seems to occur in nearly all parts of the plant

although high background signal in stamens and in the areas of active cell division cannot be excluded. This ubiquitous expression is also similar to that of RGA (Silverstone AL *et al*., 1998) in *Arabidopsis*.

The *in situ* hybridization experiments indicate that the spatial expression of *AmGRAS* is higher in the reproductive organs, stamens and ovules. In stamens, the *AmGRAS* mRNA is primarily localized in sporogenous tissue Fig. 23, B (indicated by arrow), which is complementary to *DEF* expression. *DEF* expressed in the filament and the connective (Fig. 23, A). In female reproductive organs, the AmGRAS expression is observed in ovules (Fig. 23, B). The expression of *AmGRAS* in reproductive organs is very similar to that of *RSI*, supporting a possible *in vivo* interaction of both proteins, as observed in *in vitro* experiments.

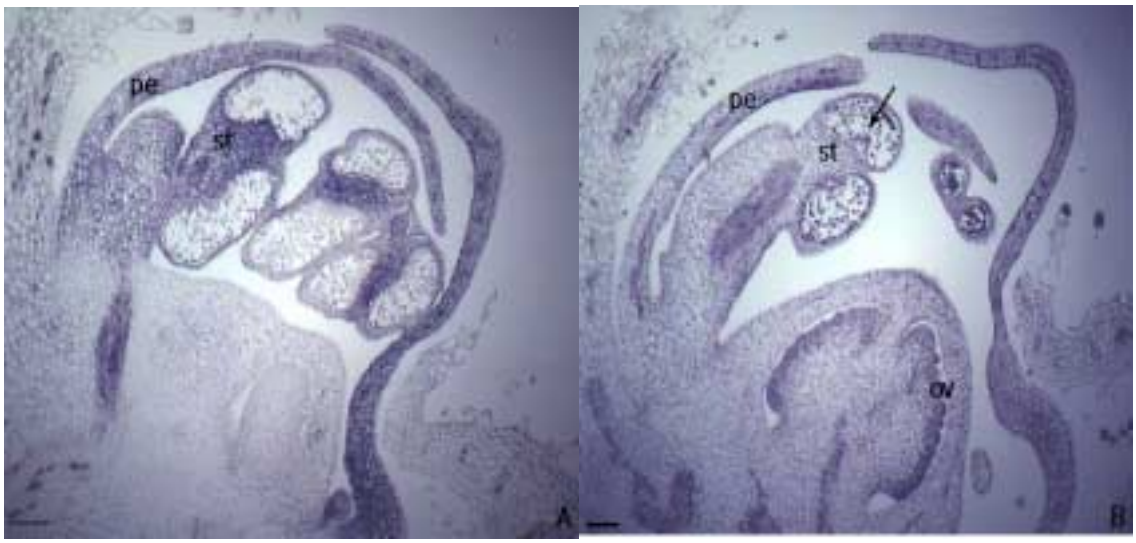

Fig. 23. *In situ* hybridization with *DEF* and *AmGRAS* probes. Longitudinal sections of young floral buds were hybridized with either an antisense *DEF* riboprobe (A) or an antisense *AmGRAS* riboprobe (B). Antisense and sense riboprobes were labeled with digoxigenin-11-rUTP using the Boehringer nucleic acid labelling kit. DNA templates were either cDNAs subcloned into pBluescript SK and prepared by PCR using T3/T7 primers. T7 RNA polymerase was used to synthesize antisense DIG-RNA.
pe: petal, st: stamen, ov: ovule. The arrow in B indicates sporogenous tissue.
Scale bars in (A) and (B) are both 100 μm.

3.9 Generation of *AmGRAS* knock-out plants by the Engrailed technique

There are two distinct classes of repressors, one is passive and the other is active. Passive repressors would directly interfere with the binding of activators, while active repressors could counteract transcriptional activators at a distance with separable DNA binding and effector domains. Many higher eukaryotic transcription factors have been found to have such an active repressor domain (Jaynes, *et al*., 1991). One well-characterized protein is EN (Engrailed) from *Drosophila*, containing a homeodomain related in DNA binding specificity to that of members of the *Antennapedia* class. By swapping homeodomains between FTZ (*fushi tarazu*), a member of the *Antennapedia* class of transcriptional activations, and EN, it was shown that EN was counteracting to the function of the endogenous FTZ protein, to generate a *ftz* mutant phenotype in embryos (Jaynes, *et al*., 1991; Tolkunova *et al*., 1998).

To generate transgenic knock-out plants of *AmGRAS*, the AmGRAS ORF was fused to the engrailed domain (EN) to produce a 35S::EN::*AmGRAS*::pGPTV-BAR construct (Fig. 24) which was for plant transformation (Dr. W. Werr, personal communication). A schematic diagram indicates the insertion of the 35S::EN::*AmGRAS* cassette into the binary vector between the right and the left T-DNA border. The binary vector, pGPTV-BAR (Becker *et al*., 1992), contains the phosphinothricin acetyl transferase (bar) as selectable marker, located near the left T-DNA border. The AscI linker was ligated into the SmaI site of the original pGPTV-BAR, into which the engrailed domain from pUC 19, digested with AscI, was integrated (Ueberlacker *et al*., 1996).

Fused to the engrailed domain (EN) *AmGRAS* was digested with AscI from pUC 19 and integrated into the AscI site of the pGPTV-BAR vector. The correct direction of insertion of the gene was confirmed by PCR using the gene specific primer 2Y16L4, and a primer from the pAnos region, or one from the 35S promoter region. Using this construct, plant transformation

of *A. thaliana* by vacuum infiltration was performed.

Transgenic plants are easily selected by spraying with the BASTA herbicide (Hoechst).

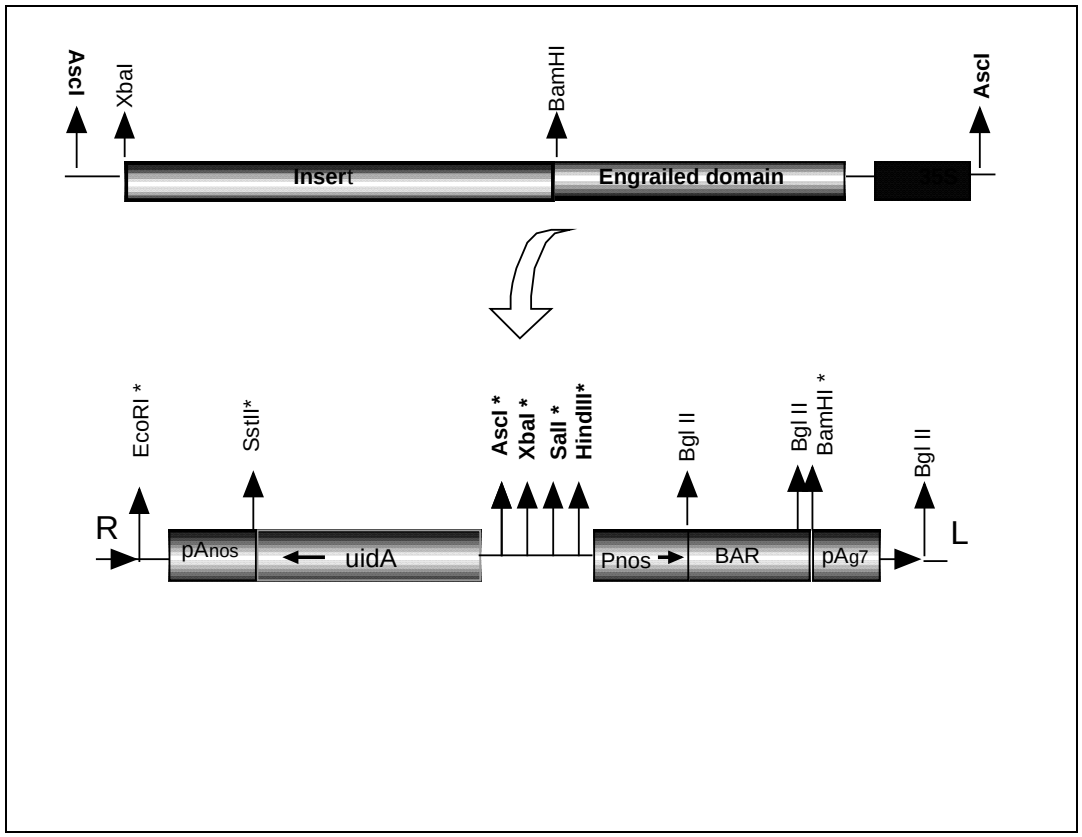

Fig. 24. Schematic diagrams of the 35S::EN::*AmGRAS*::pGPTV-BAR vector construct. It contains unique cloning sites (*) upstream of the β-glucuronidase (*uidA*) gene which allow the construction of promoter fusions. The T-DNA nopaline synthase (pAnos) and gene 7 (pAg7) poly (A) signals follow the *uidA* gene and selectable marker genes (*bar*), respectively. Arrows indicate direction of transcription; R, right T-DNA; L, left T-DNA border.

3.9.1 Phenotypes of 35S::EN::*AmGRAS* transgenic *Arabidopsis* plants

A total of 37 BASTA resistant transgenic plants were obtained. The presence of the EN::*AmGRAS* insert was checked by PCR. The overall phenotype of 35S::EN::*AmGRAS* plants is described in Table 2. Generally, no striking phenotype was observed in plants from successive generations, which were grown in long day (LD) condition of the 37 transgenic plants (Fig 25).

Table 2. Phenotype of EN::*AmGRAS* transgenic plants

generation	Phenotype
T_1	LD: no differences to wild type
	SD: dwarfism, shorter internode length, occasionally reduced numbers of petal
T_2	LD: no differences to wild type
	SD: growth retardation, partial floral organ defects*
T_3	LD: no differences to wild type
	SD: similar to T_2

LD (Long day) condition: 16rs day and 8hrs night.

SD (Short day) condition: 8hrs day and 16hrs night.

* this phenotype was obtained under poorly controlled growth condition during summer season or in a not well controlled growth chamber.

Fig. 25. Mutant phenotype of a 35S::EN::*AmGRAS* plants in LD compared to wild type in T_1 generation
A: Wild type plants
B: Trasngenic 35S::EN::*AmGRAS* plants
Transgenic plants were selected by spraying with BASTA after germination (0.1% for one-two week old plants, 0.5% for two-three week old plants and 1% for four week old plants; 0.5 l for one tray which contains 11 pots (12cm×12cm). All plants were grown in LD condition after sowing.

Several defects were only observed on plants in short day (SD) condition from the T_1 generation. These defects, which are described in the following were restored to wild type phenotype when plants were shifted to LD condition.

Three lines which were extensively analysed (in SD condition) from the T_1 generation, first showed phenotypically dwarfism compared to wild type (Fig. 26, only two lines are shown). Later in development, these lines showed shorther internode length and occasionally reduced numbers of petals (Fig. 27 B,C and D).

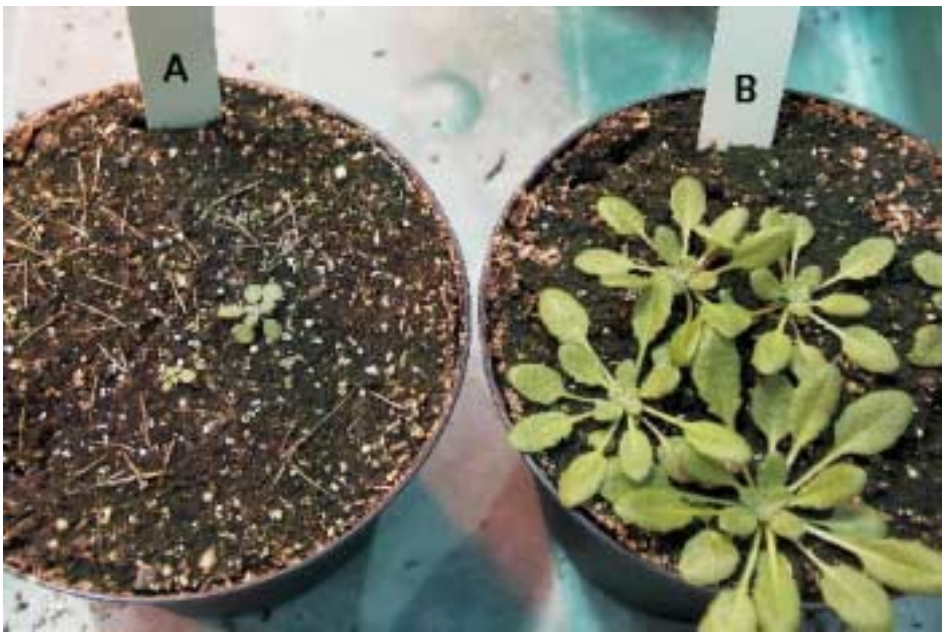

Fig. 26. Mutant phenotype of 35S::EN::*AmGRAS* plants in the vegetative stage of the T_1 generation (SD condition).

A: Transgenic 35S::EN::*AmGRAS* plants

B: Wild type plants

Transgenic plants were selected by spraying with BASTA after germination (0.1% for one-two week old plants, 0.5% for two-three week old plants and 1% for four week old plants; 0.5 l for one tray which contains 11 pots (12cm×12cm). All plants were grown in SD condition after sowing.

Fig. 27. Mutant phenotype of 35S::EN::*AmGRAS* T_1 plants after flowering in SD condition.

A: Wild type plants

B: 35S::EN::*AmGRAS* transgenic plants

C and D: 35S::EN::*AmGRAS* transgenic plants after flowering.

C is a magnified picture of B. Lower number of petals is visible in D.

Transgenic plants were selected by spraying with in BASTA after germination (0.1% for one-two week old plants, 0.5% for two-three week old plants and 1% for four week old plants; 0.5 l for one tray which contains 11 pots (12cm×12cm). All plants were grown in SD condition after sowing.

In addition to the wild type plants (Columbia ecotype), transgenic plants containing only the vector construct without *AmGRAS* were also compared with plants of the T_2 generation. The dwarf phenotype seems to be lost in the T_2 generation as compared to the T_1 generation.

However, several features of growth retardation and partial floral organ defects were still observed in almost all plants of the T_2 generation.

The floral organ defects including male sterility, ovule defects, shorter petals and shorter stamens were observed in poorly controlled constitutive SD condition or poor environmental condition during the summer (Fig. 28, B and D). These phenotypes were sometimes also observed in wild type plants. Therefore, further analysis is probably necessary to decide whether these floral organ defects are caused by environmental effects or whether they are due to the EN::AmGRAS transgene.

Fig. 28. . Mutant phenotype of floral organs of 35S::EN::*AmGRAS* plants in SD condition.

Detailed floral organ defects were visualized using light microscopy

A and C show wild type plants

B and D are transgenic 35S::EN::*AmGRAS* plants

B shows ovule abortion, and D shows that male organs are shortened and dried. D displays reduction of petal's size.

4. Discussion

4.1 Spatial and temporal control of B-function gene *DEF*

The spatial and temporal expression patterns of the floral B-function genes *DEF* and *GLO* in *Antirrhinum* throughout flower development have been characterized in several different mutants (Schwarz-Sommer *et al*., 1992; Troebner *et al*., 1992). Specially focusing on *DEF*, there are several genes, which play a key role in activation and repression of gene expression during development and which are described in the following.

4.1.1 *FIMBRIATA* (*FIM*), *CHORIPETALA* (*CHO*), and *DESPENTEADO* (*DES*)

FIM, *CHO*, and *DES* are regulators of the B-function which were analyzed extensively at the genetic level and partially at the molecular level.

fim mutants cause an overall reduction in the activity of the B-function genes, and restriction of the C domain to the innermost whorl of the flower (Simon *et al*., 1994). Its expression pattern in early stages encompasses the presumptive domain of the B- and C-function genes in the floral meristem, and in later stages rapidly expands outwards as a ring around the center of the floral meristem, adjacent to the sepal primordia. Thereafter, *FIM* is localized at the junction between sepals and petals, and between petals and stamens throughout later floral development (Simon *et al*., 1994). The overlapping mRNA expression of *FIM* with the B-function gene *DEF* in early stages is indicating that *DEF* is activated by *FIM*.

FIM contains an F-box motif and is able to interact with FIM–associated proteins which are closely related to yeast SKP1 proteins. This suggests that these complexes promote degradation of a repressor of the B- and C-function genes through the ubiquitin degradation pathway (Ingram *et al*., 1997).

CHO and *DES* were reported to be negative regulators of the expression of B- and C-function genes based on the pleiotropic phenotype of these mutants (Wilkinson *et al*., 2000).

The epistasy of *cho* over *fim* in the *cho fim* double mutant suggests that CHO is a potential target of degradation by the F-box protein (Ingram *et al*., 1997).

While *FIM* activates expression of the B-function in whorl 2 and 3, prevent *CHO* and *DES* expression in the first whorl.

4.1.2 *PLENA* (*PLE*) and *FARINELLI* (*FAR*) switch the B-function gene off in the fourth whorl

In early stages of wild type flowers, the mRNA expression pattern of *DEF* overlapps with that of the C-function gene *PLE* in the central region of the floral meristem when sepal primordia become visible, though *DEF* expression extends beyond *PLE* towards the periphery (Bradly *et al*., 1993). The *PLE* signal is more restricted than that of *DEF* to the center of the dome. In later stages, both *DEF* and *PLE* signals persist in whorl 2 and 3 and whorl 3 and 4, respectively, through to late stages of organ differentiation. The overlapping expression of *PLE* and *DEF* in the third whorl explains the positive role of *PLE* for the establishment of stamen organ identity.

In contrast to *Arabidopsis*, *Antirrhinum* has a second C-function gene. *FAR* was isolated (Davies *et al*., 1999) using the *Arabidopsis* C-function gene *AGAMOUS* (Yanofsky *et al*., 1990) as a probe. Despite strong similarities between *FAR* and *PLE*, the phenotypes of their respective mutants are dramatically different (Davies *et al*., 1999). Unlike *ple* mutants, which show homeotic conversion of reproductive organs to petaloid or sepaloid organs and a loss of floral determinacy, the *far* mutant has normal flowers which are partially male-sterile.

In the third whorl, the *ple-1* mutant contains petaloid organs which resemble stamens in

that their lower part is filament-like and narrow. However, the third whorl organs of the *ple-1 far* double mutant have more pronounced petaloid morphology which suggests that although *FAR* is unable to compensate the loss of *PLE*, *FAR* also plays a positive role in establishing stamen organ identity, like *PLE* does (Davies *et al*., 1999).

Genetic and molecular analysis of the *ple-1 far* double mutant reveals a negative control over the B-function genes in the fourth whorl (Davies *et al*., 1999). The fourth whorl of *ple-1 far* double mutant flowers consist of four to five petals, which are similar to the second whorl petals. Expression of B-function genes in *ple-1 far* mutant is markedly stronger in the second and third whorls, and clearly detectable in the fourth whorl. This ectopic expression of *DEF* and *GLO* in the fourth whorl of *ple-1 far* double mutants clearly indicates that *PLE* and *FAR* exert a negative regulatory function in wild type *Antirrhinum* flowers to prevent the expression of the B-function genes in the fourth whorl.

4.1.3 RSI, a putative repressor in the control of *DEF* expression

However, none of above genes has been shown to be a direct regulator of the *DEF* gene at the molecular level. There is a functionally important region in the *DEF* promoter, which containss several conserved motifs important for regulation of DEF gene expression (Schwarz-Sommer *et al*, 1992). Using this promoter region in a yeast one-hybrid screen as bait, a putative regulator of *DEF* was isolated, called ROSINA (RSI; M. Roccaro, personal communication).

The RSI protein contains several domains characteristic for transcription factors, including a b-ZIP domain at the C-terminal end and a serine rich region (M. Roccaro, personal communication). In floral organs, *RSI* mRNA is first detected in stamens and at later developmental stages in sepals and carpels. In later stages of stamen development, *RSI* expression is found in sporogenous tissue of anthers and ovules (M. Roccaro, personal

communication) which is complementary to that of *DEF*.

The complementary expression pattern between *DEF* and *RSI*, and the ability of RSI to bind to a region of the *DEF* promoter, suggested that *RSI* may act as a putative repressor of *DEF* activity.

4.1.4 Spatial and temporal control for stamen development

In stamens, the expression of *PLE* is found in the filament and connective and is strong in the anther wall. In later stages the mRNA of *PLE* is weak or absent from sporogenous tissue. Detailed mRNA expression analysis of *PLE* and *FAR* in reproductive organ, shows complex patterns of both genes in wild type flowers. In the anther, *PLE* transcripts become localized to the region of the stomium, whereas *FAR* transcripts are predominant in the connective (Davies *et al*., 1999). In the gynoecium, *PLE* is expressed mainly in the developing ovules and to a lesser extent in the placenta and carpel wall, whereas the expression of *FAR* is complementary to that of *PLE*.

Taken together, the expression of *DEF* in stamen is very similar to that of *PLE* and *FAR*, on the basis of strong expression in the filament and connective, and the analysis of later stages revealed that *DEF* is absent from sporogenous tissue of anthers.

To better understand the function of RSI, which has a complementary mRNA expression in sporogenous tissue of stamens compared to other genes described above, the isolation and characterization of seven RSI interactors are quite informative.

4.2 Interactors of RSI

Several candidates interacting with RSI were isolated from an cDNA expression library of *Antirrhinum majus* using a yeast two-hybrid screening.

BLAST searches were performed to identify similar proteins from different organisms in various databases (Table 1). Taking into account only highly scoring proteins from databases, three of the proteins (Y6, Y35 and 2Y6) that were isolated in the screening are homologous to database entries with unknown functions. 2Y6 and Y6, rescued five times, showed high amino acid sequence similarity to three proteins from human and *Arabidopsis*, the function of which is unknown, and Y35 also showed 85% amino acid identity with a hypothetical protein of unknown function from *Arabidopsis*.

Y13 displays 70% identity with a Ser/Thr protein kinase from *Arabidopsis*. Intracellular serine/threonine kinases are important for the transmission of signals from the membrane to cytoplasmic components and the nucleus, and for the integration of a signaling pathway. The prototype for all protein kinases, including tyrosine kinases, is PKA (Protein Kinase A). PKAs are important in the regulation of a number of physiological processes, including metabolism, cell differentiation, gene transcription and membrane transport (Beebe, 1994). 2Y14 has similarity to a RHO-associated coiled-coil forming kinase. RHO proteins are a subgroup of the RAS superfamily. RAS is known to be involved in the regulation of cellular proliferation and terminal differentiation (Hooykaas *et al*., 1999). In mammals, RAS is activated by tyrosine kinases. Some of these kinases phosphorylate the SHC protein which, together with autophosphorylated receptor proteins, then bind the SH2 domain of GRB2. The resulting complex recruits SOS, a guanine nucleotide dissociation stimulator, to the plasma membrane and SOS promotes release of GDP from inactive RAS, allowing GTP to bind and activate RAS. Active RAS can directly stimulate effector proteins further downstream in the transduction

cascade. Some of the downstream proteins are believed to include the mitogen-activated protein kinases (MAPKa) and other serine/threonine protein kinases such as RAF (Bokoch *et al*., 1993).

The finding that a Ser/Thr protein kinase and a RHO-associated coiled-coil forming kinase interacting with RSI may be an indication that *DEF* is possibly regulated via protein kinase mediated signaling. So far no experimental data have been reported about the roles of protein kinases for the growth and development of plants.

Among the interactors, 2Y16 (later renamed AmGRAS) showed similarity to GAI (Peng *et al*., 1997) and RGA (Silverstone *et al*., 1998) from *Arabidopsis*. Both proteins belong to the GRAS family of transcription factors. Genetic studies and the predicted gene functions, which will be described later, suggested that they encode repressors of GA signaling.

4.3 AmGRAS and GA signaling genes

The GRAS family proteins (formerly called VHIID proteins) which contain the VHIID domain are found in diverse plant species but not in yeast, prokaryotes, or animals. So, they are probably unique for plants, like also the AP2 family of transcription factors (Okamuro *et al*., 1997; Weigel, 1995).

AmGRAS is a new member of the GRAS family in *Antirrhinum majus*. AmGRAS has several highly conserved domains characteristic of GRAS family proteins. The PSORT program (Nakai and Kanehisa, 1992; http://psort.nibb.ac.jp) predicts that AmGRAS should localize to the nucleus, because it contains a bipartite nuclear localization signal (NLS). AmGRAS contains also a DELLA domain, two large leucine heptad regions, a VHIID domain, a LXXLL domain, a RVER domain, a SH2-like domain, and a SAW domain (Fig. 15.).

Leucine heptad repeats have been demonstrated to mediate protein-protein interactions among proteins (Hurst *et al*., 1994). AmGRAS has a LHQLL motif, which is identical to the consensus sequence LXXLL that was demonstrated to mediate the binding of steroid receptor coactivator complexes to nuclear receptors (Heery *et al*., 1997). The SH2 domain is also an indicator of protein-protein interaction, it binds phosphotyrosine containing proteins. There is also a SH2-like domain present in AmGRAS. These three domains further support the idea that AmGRAS is interacting with other proteins.

GAI and RGA are homologous genes that encode putative transcription regulators which repress GA signaling, because some aspects of growth in *Arabidopsis*, such as stem and hypocotyl elongation in plants lacking either GAI or RGA function, require less GA than those of wild-type plants.

The *GAI* (Gibberellin Insensitive) gene has been isolated by insertional mutagenesis of the gain-of-function *gai* allele (Peng *et al*., 1997). The loss-of-function (null) alleles restored the dwarfism phenotype to wild-type. The gibberellin response pathway is partially de-repressed in these plants. Thus, this gene was suggested to be a negative regulator of the GA response (Peng *et al*., 1997).

The *RGA* (Repressor of the *ga1-3* mutant) is also reported to be a negative regulator of the GA signal transduction pathway (Silverstone *et al*., 1998), because the recessive *rga* mutant is able to partially suppress phenotypic defects of the *Arabidopsis* gibberellin biosynthetic mutant *ga1-3* (Sun *et al*., 1992; Silverstone *et al*., 1997a). Defects in stem elongation, flowering time, and leaf abaxial trichome initiation are suppressed by *rga*. The *ga1-3* allele is a GA biosynthetic mutant which is a non-germinating, male-sterile, extreme dwarf blocked in the first committed step of GA biosynthesis (Koornneef *et al*., 1980). This mutant requires GA for germination and seed set.

The *rga-24 gai-t6* double null mutant in *ga1-3* background completely restores all defects which are partially rescued by *rga* except for the male sterility (Dill and Sun, 2001; King *et al*., 2001). However, the *gai-t6* null allele alone has little effect in suppressing the phenotype of *ga1-3* (Dill and Sun, 2001; King *et al*., 2001). Therefore, GAI and RGA seem to have partially redundant functions in repressing GA signaling with RGA playing a more dominant role than GAI.

It has been hypothesized that the GA signal may inhibit GAI function by interacting directly or indirectly with the DELLA sequence, because the semi-dominant *gai-1* mutant which has a deletion in the DELLA domain resembles GA-deficient mutants, with the difference that it cannot be rescued by GA treatment (Koornneef *et al*., 1985; Peng *et al*., 1997). The functional homologs of *GAI* and *RGA* in several crop plants, such as *Rht* in wheat, *D8* in maize, and *SLR* in rice (Ogawa *et al*., 2000; Ikeda *et al*., 2001) have been isolated. Deletions of the DELLA region in these genes also confer a similar semi-dominant dwarf phenotype in these crop plants.

Expression of *GAI* and *RGA* is only slightly affected in different GA response mutant backgrounds or by GA treatement. However, the endogenous RGA protein levels are dramatically reduced after GA application. Therefore, it was hypothesized that the GA signal seems to derepress the GA signaling pathway by degrading the repressor protein RGA (Dill *et al*., 2001).

The functional homolog of GAI and RGA in *Antirrhinum* is very likely AmGRAS which is closer related to GAI than to RGA at the protein level, with higher amino acid identity. It contains a DELLA domain, which could suggest that AmGRAS is also involved in GA signaling, probably having a similar function as GAI or RGA in *Arabidopsis*.

4.4 RSI as an interactor of AmGRAS

4.4.1 The role of gibberellin signaling in flower development

Plant hormones like gibberellins (GAs) and auxins modulate growth and development in response to both endogenous and environmental signals. Gibberellins were first discovered in the 1930s, as a metabolite produced by a pathogenic fungus (*Gibberella fujikuroi*) that caused excessive elongation of rice stems (Takahashi, *et al* 1990).

GAs, which are tetracyclic diterpenoid growth factors, control a variety of plant growth and developmental processes including seed germination, stem elongation, flower initiation, and flower and fruit development (Hooley, 1994). GAs are very important in agriculture. World crops yields were substantially increased in the 1960s and 1970s, because farmers rapidly adopted the new varieties and cultivation methods of the so-called green revolution. The new varieties are shorter, have increased grain yield and reduced straw biomass, and are more resistant to damage by wind and rain. Although the GA biosynthetic pathway has been well characterized biochemically, until now little is known about GA action even though several GA response mutants which cause characteristic features of the new varieties, have been isolated. Several of these genes which encode GA response modulators belong to the GRAS family (Peng *et al*., 1999).

To gain deeper insight into GA signal transduction, *SPY* (Jacobsen *et al*., 1996), *GAI* (Peng *et al*., 1997) and *RGA* (Silverstone *et al*., 1998), were extensively studied with genetic and biochemical methods. The original *spy* mutants were isolated based on their ability to germinate in the presence of the GA biosynthesis inhibitor paclobutrazol (Jacobsen and Olszewski, 1993). The cloning of SPY provided information about the protein which contains tetratricopeptide repeats in the deduced amino acid sequence, the function of which is to

mediate protein-protein interactions. These characteristic repeats occur in a diverse range of proteins (Jacobsen *et al*., 1996).

Recently several cloned Ser (Thr)-O-GlcNAc transferases were shown to be homologous to SPY (Kreppel *et al*., 1997; Lubas *et al*., 1997). These glycosyltransferases play important roles in regulating the activities of various nuclear and cytosolic proteins. These enzymes can modify proteins by glycosylation alone or by competing for phosphorylation sites. The modified sites are typically rich in Ser and Thr, and both RGA and GAI have such a region at their N-termini. Based on the biochemical function of these genes, it was proposed that SPY might activate RGA and GAI by GlcNAc modification. A second enzyme is required for removing the GlcNAc residue.

Two other genes, *GAI* and *RGA*, are involved in GA signal transduction. From genetic studies, it was shown that the *spy* mutant is epistatic to the *gai* mutant. Concerning *SPY* and *RGA*, there are additive effects between the *spy* and *rga* mutants in suppressing the defects of the *ga1-3* mutants (Jacobsen *et al*., 1996; Silverstone *et al*., 1997). Because RGA and GAI are functionally not completely redundant and do not contain well-defined DNA-binding domains, it was suggested that possibly other interacting or modifying proteins are required which are specific for either RGA or GAI. Due to these functional features of the two proteins, it was intriguing to search for interacting partners of the GRAS proteins.

4.4.2 Are GRAS proteins functional homologues of STATs ?

The GRAS family proteins were suggested to be functionally related to the STATs (Signal Transducers and Activators of Transcription) of mammals, since they have several important structural features in common with the STATs, and the arrangement of those features in the two protein families is the same (Richards *et al*., 2000). They have a divergent N-terminal region,

leucine heptad repeats, a DNA-binding domain, and a SH2 domain, although the DNA-binding domain and the SH2 domain are not yet clearly defined for the GRAS proteins.

STATs are known in many non-plant species, where they act as intracellular intermediaries between extracellular ligands and the transcriptional activation of genes (Darnell, 1997). STATs form homo- or heterodimers, are activated by phosphorylation, translocate to the nucleus, and bind to DNA, thus regulating transcription of genes.

In the STATs, two conserved amino acid motifs were shown to be involved in DNA binding (Horvath *et al*., 1995). One is the sequence VX(E,D)E, the other is a motif containing several valines (LPVVV/II). GRAS proteins also contain a valine rich motif (VVLV, between amino acids 148-151 in AmGRAS) which is very similar to the sequence LPVVV/II in STATs. In addition they contain a VHIID domain, the first motif identified to be common to all GRAS proteins. Although there are putative DNA-binding domains in GRAS proteins which are similar to those of STATs, no biochemical evidence for DNA binding has been reported so far.

In *Antirrhinum*, AmGRAS is an interactor of RSI, which binds to the *DEF* promoter. RSI in the AmGRAS/RSI complex could mediate DNA-binding of the heterodimers and thus connect signal transduction with transcriptional gene activation. Thus, the interaction of AmGRAS with RSI is perhaps a step forward to understand the role of the GRAS proteins in flower development by GA signaling and their relationship with STATs.

At the molecular level, little is known about how GAs regulate flowering, but there are some genes known which are regulated by GA during flowering. *LEAFY*, a floral meristem-identity gene in *Arabidopsis*, is known to regulate early floral events, and its promoter is responsive to GA (Blazquez *et al*., 1998). In addition, applied GA can rescue the weak flowering phenotype of *leafy* mutants (Okamuro *et al*., 1996). A further candidate gene is the GAMYB transcription factor that is specific to the GA signal transduction pathway in

flowering, especially floral transition of the shoot apex (Gocal *et al*., 1999). The *LEAFY* promoter contains a potential MYB-binding motif that is required for normal *LFY* promoter activity (Blazquez and Weigel, 2000). The *AmGRAS* promoter also contains putative MYB binding core motifs, GTTA. It might be interesting to test whether these core motifs in the *AmGRAS* promoter are really functional in flower development. Further biochemical and genetic studies of these roles of AmGRAS and RSI in floral organ development will shed light on the control of floral B-function and GA singal transduction.

The SH2 domain, present in STATs family, was shown to mediate the binding of STATs to various receptor tyrosine kinases, which then gets activated by the receptor kinase. Interestingly, there is a putative sequence-specific binding motif for SH2 domain proteins in the amino acid sequence of RSI between amino acids 142 and 156, G-VYENDD-VYENDD-G (M. Roccaro, personal communication). AmGRAS contains a SH2-like domain (Fig. 15). This putative binding site was identified based on the specific phosphopeptide sequence recognized by SH2 domains (Songyang *et al*., 1993). The tyrosine (Y) present in this RSI motif might be phosphorylated and could be a putative target of SH2 proteins.

Since AmGRAS contains a SH2-like domain, the interaction with other RSI interactors, like a Ser/Thr protein kinase and RHO-associated coiled-coil forming kinase, which were thought to be downstream proteins of RAS, was tested. No interaction between these RSI interactors and AmGRAS when AmGRAS was used as bait in the yeast two-hybrid system.

If one can show that the SH2-like domain in AmGRAS is functional, by using the binding motif of RSI, it will support the idea that the GRAS proteins are plant STATs and will contradict the idea that the SH2 domain is exclusively associated with the evolution of multicellularity in animals (Schindler *et al*., 1995).

4.5 *AmGRAS* is a putative orthologue of *GAI* or *RGA*

The phylogenetic comparison of AmGRAS with other GRAS family proteins shows that AmGRAS is closely related to the GAI and RGA proteins from *Arabidopsis*. AmGRAS shares 66% identity with GAI and 59% identity with RGA at the amino acid sequence level.

The growth of *rga-24 gai-t6* double mutant in a wild type background is similar to that of wild type plants. However, although there are no big differences in the plant height compared to that of wild type plants, there are some differences between the *rga-24 gai-t6* double mutant and wild type plants. The *rga-24 gai-t6* mutant has much reduced fertility (Dill and Sun, 2001; King *et al*., 2001). The double mutant has shorter siliques producing fewer seeds, reduced amounts of pollen and the stamen filaments are shorter than the carpels.

The *rga-24*, *gai-t6*, and *rga-24 gai-t6* mutant allele in the *ga1-3* mutant background do not restore the defects in germination and flower development as reflected in the nongerminating, male-sterile phenotype of *ga1-3*. The *rga-24 gai-t6 ga1-3* triple mutant flowers have severely reduced petal and stamen growth and are sterile. The failure to rescue several defects regarding germination and flower development in *rga-2 ga1-3* and *gai-t6 ga1-3* double or *rga-24, gai-t6 ga1-3* triple mutant suggests that there are additional genes in functionally redundant ways involved. These genes could be *RGL* (*RGA-LIKE*; Sanchez-Fernandez *et al*., 1998), *RGA1-LIKE* (Genebank accession No. AC009895), and *RGA-LIKE PROTEIN* (Genebank accession No. AL391150). All of them contain a DELLA domain with 56-60% amino acid sequence identity to GAI and RGA (Dill and Sun, 2001).

Although the double and triple mutants in a *ga1-3* background do not tell the functional roles of *GAI* and *RGA* in flower development, there is some indication of the roles of *GAI* and *RGA* from the phenotype of the *rga-24, gai-t6*, and *rga-24 gai-t6* alleles in a wild-type background (Dill and Sun, 2001; King *et al*., 2001). The fertility defects in reproductive organs

of the *rga-24 gai-t6* double mutant are consistent with the spatial expression pattern of *AmGRAS* (Fig.22 and 23). The floral defects of *AmGRAS* transgenic plants grown in SD condition are very similar to those of the *rga-24 gai-t6 ga1-3* triple mutant (Fig. 28), although these *AmGRAS* transgenics have to be analyzed more in detail.

Taken together, all this indicates that *AmGRAS* is functionally closer to *GAI* and/or *RGA*, and suggests that *AmGRAS* is the *Anrirrhunum* orthologue of *GAI* and *RGA* of *Arabidopsis*.

4.6 Overlapping expression patterns of *AmGRAS* and *RSI* in stamens

AmGRAS is more or less ubiquitously expressed in nearly all plant organs, including sepals, petals, stamens, carpels and leaves. The mRNA expression pattern of *AmGRAS* was dectected by *in situ* hybridization in several different tissues such as inflorescence apical meristems, floral meristem, and flower organ primordia.

It is interesting that the expression of *AmGRAS* is differentially higher in the reproductive organs compared to others. In stamens, the *AmGRAS* mRNA is more localized in sporogenous tissue which is complementary to *DEF* expression (Fig. 23). In the female organ, AmGRAS expression is primarily in ovules (Fig. 23, B). The expression of *AmGRAS* in reproductive organs is very similar to that of *RSI*. The similar spatial expression patterns of *AmGRAS* and *RSI* clearly supports the interaction of both proteins in the yeast two-hybrid system and in GST pull-down experiments, and indicates its biological relevance.

4.7 Possible roles of AmGRAS and RSI for the regulation of *DEF*

Transgenic knock-out plants of *AmGRAS*, using the Engrailed domain did not give a clear answer about the functional roles of this gene, although the plants showed several defects in the floral reproductive organs. However, the expression pattern of *AmGRAS* and *RSI* is complementary to that of *DEF*. Based on the similar expression patterns of *RSI* and *AmGRAS* and the interaction of the encoded proteins, it is reasonable to speculate that the heterodimer is involved in the control of *DEF* gene activity, thereby linking GA-signaling and activation of the floral B-function.

It is also possible that the AmGRAS/RSI heterodimer participates in the regulation of other genes and developmental pathways in flower development.

4.8 Perspectives

The work described in this thesis allowed the identification of AmGRAS, which might be involved in the GA signaling pathway in flower development. So far the evidence is preliminary and has to be substantiated. Whether AmGRAS is playing a STAT-like role in signaling in plants, is an interesting question, which should be investigated. For this biochemical analysis of the putative interaction of the SH2-like domain in AmGRAS with the putative sequence-specific binding motif for SH2 domain proteins in RSI should be informative.

The similar mutant phenotype of floral organs of *AmGRAS* transgenics compared to that of the *rga-24 gai-t6 ga1-3* triple mutant is probably due to the fact that other genes of the GRAS family are also knocked out. To identify the specific function of *AmGRAS*, other constructs have to be designed using only specific parts of the ORF, such as the more divergent N-terminal region of GRAS proteins, which could be analyzed in transgenic plants in *Arabidopsi*s

and *Antirrhinum*. The specific function of *AmGRAS* in transgenic knock-out plants can be checked by RT-PCR, and also the question whether other *GAI* or *RGA*-like genes are intact or also inactivated.

The result from these experiment possibly will not only contribute to the understanding of the role of AmGRAS/RSI in *DEF* control but also shed light on various aspects of GA signaling in flower development.

5. Summary

In *Antirrhinum*, development of petals and stamens in the second and third whorl is controlled by the homeotic B-function genes *DEFICIENS* and *GLOBOSA*, which belong to the MADS-box family of transcription factors. The encoded proteins form heterodimers via their K-domain, which control petal and stamen organogenesis. According to the genetic ABC model, these two genes establish the B-function, which, together with the A- and C-functions, regulate floral organ development. The spatial and temporal expression patterns of *DEF* and *GLO* is not uniform during this process. In early stages of development transcripts of *DEF* are detectable in the second and third whorl primordia. In later stages, *DEF* transcripts are found also in first and fourth whorl organs. *GLO* mRNA is found mainly in the second- and third whorl primordia, but later on also in fourth whorl organs (Schwarz-Sommer *et al*., 1992; Troebner *et al*., 1992; Zachgo *et al*., 1995). The analysis of expression patterns of *DEF* and *GLO* in mutants suggests that they are independently induced and highly regulated. Indeed, several genes are known to be required for this process, as deduced from genetic analysis of *Antirrhinum* mutants, but little is known about the molecular mechanisms that control the B-function genes *DEF* and *GLO*.

A first step for a better understanding of the molecular control mechanisms was the isolation of *ROSINA (RSI*), a putative regulator of *DEF*, in a yeast one-hybrid screen (M. Roccaro, pers. Communication), using a 200 bp long *DEF* promoter fragment which contains several potential binding sites for transcription factors like b-ZIP- and MADS-box proteins. In addition, a three bp deletion in this region in the mutant allele *deficiens-chlorantha (def-chl)* leads to a strong reduction of *DEF* expression in the second and third whorl, phenotypically visible as partial homeotic changes of the petals and stamens (Schwarz-Sommer *et al*., 1992).

RSI is a member of the b-ZIP family of transcription factors which contains, besides the basic domain and the leucin zipper, several other domains of uncertain function.

Since such factors often need partners for exerting their regulatory function as heterodimers or multimers, RSI was used as 'bait' in a yeast two-hybrid system to search for such potential partners. With this strategy several candidates interacting with RSI were isolated and the DNA sequence determined. A data bank search revealed homologies to known proteins like Ser/Thr kinases or transcription factors from plants and mammals, but also to proteins of unknown functions. The most interesting candidate was clone 2Y16 (later renamed 'AmGRAS') which showed strong similarity to members of the GRAS family (GAI and RGA) of *Arabidopsis*. This candidate was chosen for further analysis. The protein-protein interaction between AmGRAS and RSI was confirmed also biochemically by GST-pull down experiments. Expression studies of *AmGRAS*, by mRNA northern and in situ hybridisation, revealed great similarity to the expression patterns of *GAI* and *RGA* of *Arabidopsis*, which were suggested to be transcription factors involved in gibberellin signal transduction. For further functional characterisation of *AmGRAS* by 'knock-out' stategies, transgenic AmGRAS plants in *Arabidopsis* were made, using the 'engrailed method' of gene inactivation (W. Werr, pers. communication). Transgenic plants with mutant phenotypes were obtained and preliminary characterised, but a more careful analysis is not completed yet.

The current results and data suggest that *AmGRAS* is the *Antirrhinum* orthologue of one of the two *Arabidopsis* GRAS family members *GAI* and/or *RGA*. Since it is well known that gibberellins (GAs) play an important role in floral organ development, the protein interaction between RSI and AmGRAS could be the link which connects GA signal transduction and expression/regulation of the floral B-function gene *DEFICIENS*.

5. Zusammenfassung

In *Antirrhinum majus* (Löwenmäulchen) wird die Entwicklung der Petalen und Stamen, der Blütenorgane des zweiten und dritten Wirtels, von den homöotischen Genen *DEFICIENS (DEF)* und *GLOBOSA (GLO)* gesteuert. Die von den beiden Genen kodierten Proteine sind Transkriptionsfaktoren, die zu der MADS-Box Familie gehören und die als Heterodimere die Organogenese von Petalen und Stamen kontrollieren. Nach dem genetischen ABC-Modell der Blütenentwicklung etablieren die beiden Gene die B-Funktion, die im Zusammenwirken mit der A- und der C-Funktion die Entwicklung der floralen Organe im zweiten beziehungsweise dritten Wirtel steuern. Die Expression der beiden B-Funktionsgene ist zeitlich und räumlich veränderlich. In frühen Stadien sind *DEF* und *GLO* in den Primordien exprimiert, aus denen sich später die Organe des zweiten und des dritten Blütenkreises - Petale und Stamen - entwickeln. In späteren Entwicklungsstadien der Blüte sind beide auch im Karpel des vierten Wirtels - wenn auch schwächer als in den Organen zweiten und dritten - exprimiert, und *DEF* auch schwach in den Sepalen des ersten Wirtels (Schwarz-Sommer *et al*, 1992; Tröbner *et al*,1992; Zachgo *et al*, 1995). Die Analyse der Expressionsmuster in DEF- und GLO-Mutanten ergab, daß beide Gene unabhängig von einander angeschaltet werden und danach organ- und gewebespezifisch streng reguliert werden (Tröbner *et al*, 1992). Über die molekularen Mechanismen dieser Regulation ist bislang wenig bekannt.

Ein erster Schritt zum besseren Verständnis der molekularen Kontrollmechanismen war die Isolierung von *ROSINA (RSI)*, eines möglichen Regulators von *DEFICIENS*, mittels des One-hybrid Systems in Hefe (M. Roccaro, pers. Mitteilung). RSI wurde isoliert mit einem 200 Bp langen Promotorfragment des *DEF*-Gens, das mehrere potentielle Bindemotive für Transkriptionsfaktoren wie z.B. bZIP- und MADS-Box Proteine enthält. Außerdem führt eine

Deletion von drei Basenpaaren in dieser Region in dem Mutantenallel *deficiens-chlorantha (def-chl)* zu einer starken Reduktion der Expression im zweiten und dritten Wirtel, die phänotypisch als partielle homöotische Änderungen der Petalen und Stamen sichtbar wird. RSI ist ein Mitglied der b-ZIP-Familie von Transkriptionsfaktoren, das neben der basischen Region und dem Leucin-Zipper noch andere Domänen mit unbekannter Funktion enthält (M. Roccaro, pers. Mitteilung).

Da solche Faktoren für die Ausübung ihrer regulatorischen Funktion meistens Partner benötigen, mit denen sie dimere oder multimere Proteinkomplexe bilden, wurde RSI in einem Hefe-Two-hybrid System als "Bait" benutzt, um potentiellen Interaktoren zu isolieren. Mit dieser Strategie wurden eine Reihe (sieben) von interagierenden Kandidaten isoliert, die zunächst durch verschiedene Kontrollexperimente in Hefe bestätigt und deren DNA-Sequenz dann ermittelt wurde. Eine Suche in verschiedenen Datenbanken zeigte in einigen Fällen Homologie der RSI-Interaktoren zu bereits bekannten Proteinen wie Ser/Thr-Kinasen oder Transkriptionsfaktoren von Pflanzen (Arabidopsis) und Tieren, aber teilweise auch zu Proteinen mit unbekannten Funktionen. Der interessanteste Kandidat war 2Y16 (später umbenannt zu "AmGRAS"), der starke Ähnlichkeit zu Mitgliedern (RGA und GAI) der GRAS-Familie von Arabidopsis zeigte und der daher für eine tiefergehende funktionelle Analyse ausgewählt wurde. Die Protein-Interaktion mit RSI wurde biochemisch durch "GST-pulldown"-Experimente bestätigt. Expressionsanalysen mit mRNA Northern und in situ Hybridisierung zeigten große Ähnlichkeiten zwischen *AmGRAS* und *RGA* und *GAI* von Arabidopsis, von denen vermutet wird, daß sie Transkriptionsfaktoren sind, die in der Gibberellin-Signal-Transduktion involviert sind. Zur weiteren funktionellen Charakterisierung von AmGRAS wurde die "Engrailed-Methode" (W. Werr, pers. Mitteilung) benutzt, um mit

AmGRAS "Knock-out"-Mutanten in Arabidopsis zu erzeugen, deren Analyse allerdings noch nicht beendet ist.

Die bisherigen Ergebnisse und Daten legen die Vermutung nahe, daß *AmGRAS* das Antirrhinum-Ortholog einer der beiden Arabidopsis-Gene - *RGA* oder *GAI* - ist. Da bekannt ist, daß Gibberelline (GA) eine wichtige Rolle spielen in der Entwicklung der Blütenorgane, könnte die Interaktion von RSI und AmGRAS einen der Verbindungspunkte von GA-Signaltransduktion und der Expression/Regulation des floralen B-Funktionsgens *DEFICIENS* darstellen.

6. References

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7. Amino acid sequence of putative RSI interactors

7.1.1 Y6

MAQTIENSEFSLQERNVELEKELRNSLEREEWMKQELQKAYQRLRVAEEAEERLCLQL
GELEAEAVDHAREYRAHVMELMEQIAFAQKLLQQTSVDDISFPNSD

7.1.2 Y35

LRLRLRLRLREREIMMSSADRHSSSLSAPHHPDDNNNNNNLFLDILQDAPLFGHRKPTI
LVGSIFYCLLLTGYAILAVGAAWILQPVQELVALILCSSDVILLLVTGIFQQYLVYQVQK
IRLQGYYGFSQKLKHIIRLPFATIAYGTAAMLLIMAWKPHISILSISMLLRITMLVQAICA
GFFMSIYIGYVHQYNSLDSQPDVLNSLYSPLQQSSSLEGSRYHDGGRLSDQQMALLQY
QRENLNFLSEEILRLQECLSKYERSDDGSTPQVDLAHLLAARDQELRTLSAEMNQLQSE
LRLARSLIAERDMEIQRVRITNNQYVEENERLRAILGEWSARAAKLERALEAERMSNL
ELQKNITTLKSQTMQEQVEPKSQMDH

7.1.3 Y13

RQDGQPNHNDDYDLTDDGLFASGPQEPEFAANEDSAAIYDELVINDNKNDDYEIFELRI
IHRKNRTGFEEHKEFPIVLNSIIAGRYYVTEFLGSAAFSRVVQAHDLYSGMDVCLKIIKN
DKDFFDQSLDEIKLLKFVNKHDPADERHILRLYDYFYHQEHLFIVTELLRANLYEFQKF
NRESDGELYFSMHRLQVITRQCLEALSYLHDLGIIHCDLKPENILIKSYRRCEIKIIDLGSS
CFETDHLSLYVQSRSYRAPEVMLGLAYDQKIDLWSVGCILAELYSGEVLFPNEAVVLL
LARMVGLLGPIDMDMLRKGQEVHKYFTKEFDLYHVNEDTNQLEYIIPEESSLEDHLQI
LDPLFIDFLKYLLEINPKRRPTAREALVHPWLSHSY

7.1.4 2Y6

EFGTSGTREKETIRDSKSESFELIKRLEFHIKTLSEVHEQDKKHIEELERELSNCCQEIDY
LQDQLKIRNSDLNCLVEQVSSLQLKLADMDNLAEETESLREHVKISEYERSLLMQDIED
KEVAIRYSASRVEELEESISSIGLEFQCEIESTKLESMALEQKLFEIKELLHERTQESSRM
HELIQDLESQIRDANKVIEGLDKENKDFREKLQRRIKCQCICQEVEDQFDDRVQHKC

7.1.5 2Y14

EFGTRSSIFALDREKSKGLEEICDLLKSERSYLLTERSSLALKLENVERKLEGMEKRYM
GLEQKYADVEKEKDAMHGKVVELMFSLGMEKQERTSSQLQSETRLAGLENQIHLLQQ
DNRWKKKEFEEELEKALKAQFEISILQKFMKDMEEKNYALITECQKHVEASKLAEKLI
SELESESLEQQVESELLLDEN

7.1.6 2Y16

MKRDSSMNNNNNNNQAEQKSSSSKNMWPSSSCEATDEVDELFAVLGYKVKPSDMAD
VAIKIQQLEQVMGNGAAVSDLASDTVHYNPSDLSSWLESMITGLNQFDPPPPTQMDFG
SDLVAIPGEAAMYPQPPPIKKLKTTPHQEQPPKVVLVDSQENGVRLVHTLMACAEAVQ
QENFKLAETLVKNIGFLAVSQVGAMRKVATYFAEALARRIYRLYPTSNLQDSAFTDLL
QMHFYETCPYLKFAHFTANQAILEAFAGKTRVHVIDFSMKQGMQWPALLQALALRPG
GPPSFRLTGVGPPSPDNTDHLQEVGWKLAQLAESINVEFEYRGFVANSLADLNASMFD
VREGETVAVNSIFELHQLLARGGAIEKVLGVVRELKPEILTVVEQEANHNGVAFLDRFT
ESLHYYSTLFDSLESCGGGVEGGVVSDQDKVMSEVYLGRQICNVVACEGVDRVERHE
SLVQWRTRFNGAGFKPVHLGSNAYKQASMLLALFAGGDGYRVEENDGCLMLGWHT
RPLIATSAWRPS

7.1.7 2Y19

EFGTSGTSVTERTLEQESPQYQQLNTVLPVNETDEKVDCMVQAVDESKSLVPERQNAD
PCAEISYKSLYIESQKRIEELMENNFDLVRKLDFAHGKIEAYEKMMVPAKEVILVSDQG
KATEATVSLSPQEVQRPISQIGAAADRILSTKRKKINSKVAADATEGHNASPKQKKKYT
KRKSN

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