ROSINA (RSI), a novel protein with DNA-binding capacity, acts during floral organ development in *Antirrhinum majus*

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Summary

Petal and stamen identity of the *Antirrhinum majus* flower is under the genetic control of the floral homeotic gene *DEFICIENS* (*DEF*). To isolate factors involved in the regulation of *DEF* gene activity, a promoter segment of this B-function gene, containing *cis*-acting regulatory elements, was used to identify the novel *trans*-acting factor ROSINA (RSI). RSI does not show an extended similarity with any gene product present in the database. Rather RSI constitutes a protein that contains domains similar to known proteins from organisms of different phyla. The capacity of RSI to bind a sequence element of the *DEF* promoter, its spatial and temporal expression pattern together with the phenotype of *RSI*-RNAi interference plants as well as *RSI* over-expression in *Arabidopsis thaliana* suggest that RSI is a putative regulator of *DEF* gene activity in *A. majus*.

Keywords: *DEFICIENS*, yeast one-hybrid, DNA-binding protein, regulator, aberrant transcript, *Antirrhinum majus*.

Introduction

The flower of *Antirrhinum majus*, like those of many other eudicotyledoneous plants, is composed of four highly specialized types of organs arranged in four concentric rings or whorls. Five sepals develop in the first outermost whorl, while in the second whorl, five petals form in alternate positions to the sepals. The petals are fused for part of their length to form the corolla tube, while the upper parts form the petal lobes. Four stamens, the male organs, and one stamenoid (aborted stamen) develop in the third whorl. In the fourth innermost whorl, two fused carpels with a bilocular ovary at their base form the female organ of the flower.

The identity of each type of floral organ is under genetic control of a group of homeotic genes, several of which belong to the MADS-box family of transcription factors (Becker and Theissen, 2003; Irish, 2003; Sommer *et al.*, 1990; Yanofsky *et al.*, 1990). Mutations that functionally inactivate these genes lead to homeotic alterations of organs in two adjacent whorls. Based on these observations, a model was proposed for the establishment of floral organ identity in *Arabidopsis thaliana* (Coen and Meyerowitz, 1991; Weigel and Meyerowitz, 1994). The model postulates three developmental functions (A, B and C) specified by three classes of homeotic genes A, B and C, respectively. These genes, active

in two adjacent whorls, act either alone or in combination to establish the identity of the four floral organ types. However, A-function genes that give the same type of homeotic transformation as reported for *A. thaliana* (Bowman *et al.*, 1989; Irish and Sussex, 1990), have neither been identified in *A. majus* nor in *Petunia hybrida*, thus casting some doubts on the general validity of the ABC model (Schwarz-Sommer *et al.*, 1990; Maes *et al.*, 2001; Vandenbussche *et al.*, 2004).

Nevertheless, in *A. majus*, like in *A. thaliana*, the B-function comprises at least two genes, *DEFICIENS* (*DEF*) and *GLOBOSA* (*GLO*), which together control petal and stamen development. Mutations in either gene result in homeotic conversion of second whorl petals to sepals and of third whorl stamens to a carpeloid structure with five loculi filled with ovules. The fourth whorl is missing as a result of premature termination of floral meristem proliferation (Schwarz-Sommer *et al.*, 1992; Sommer *et al.*, 1990; Tröbner *et al.*, 1992).

DEF and *GLO* both encode MADS-box proteins that, interacting with each other to form a heterodimer, are capable of binding specific DNA motifs termed CArG-boxes (Davies *et al.*, 1996; Tröbner *et al.*, 1992; Zachgo *et al.*, 1995). In addition, DEF and GLO seem to control the maintenance

of their own expression via an auto-regulatory feedback mechanism (Schwarz-Sommer *et al.*, 1992; Zachgo *et al.*, 1995), while their initial induction is controlled by an as yet unknown mechanism.

The activity of the *DEF* and *GLO* genes is maintained throughout floral development, but their spatial and temporal expression patterns in petals and stamens change in a complex manner during the development of these organs. In wild-type flowers, both *DEF* and *GLO* transcripts are initially detected in regions of the meristem where petal and stamen primordia form (Schwarz-Sommer *et al.*, 1992; Tröbner *et al.*, 1992; Zachgo *et al.*, 1995). At later developmental stages their expression becomes differentially regulated and restricted to specific tissues and cell types. For instance, from stage 6 of floral development onwards, *DEF* and *GLO* expression is suppressed in developing sporogenous tissue of the anthers, and a differential *DEF* mRNA distribution is also seen in petals (Schwarz-Sommer *et al.*, 1992; Tröbner *et al.*, 1992; this study). The underlying molecular mechanisms of this modulation of gene expression and of its initiation at early stages are currently unknown. To identify and isolate *trans*-acting factors that control *DEF* expression we have carried out a yeast one-hybrid screen using the *DEF* promoter as bait.

Here we report the isolation of a novel gene, *ROSINA* (*RSI*), which encodes a protein that binds to a specific sequence element within the *DEF* promoter. The spatial and temporal expression pattern of *RSI*, its DNA-binding capacity, and the phenotype of *RSI*-RNAi interference plants as well as *RSI* over-expression in *A. thaliana*, suggest that RSI acts as a direct regulator of *DEF* gene activity in *A. majus*.

Results

The topology of the DEF promoter

Modulation of *DEF* expression is likely due, at least in part, to *trans*-acting factor(s) able to bind to its promoter. A region of *cis*-regulatory elements within this promoter might be defined by a mutation in the *def-chlorantha* allele that confers a mutant floral phenotype (Schwarz-Sommer *et al.*, 1992). The *chlorantha* mutation is located in a region of the *DEF* promoter that is highly conserved in the promoter of *Misopates orontium DEF* orthologue (J. Kim and E. Loennig, Max Planck Institut für Züchtungsforschung, Köln, Germany, personal communication), as well as in the promoter of the *Solanum tuberosum DEF* orthologue (Garcia-Maroto *et al.*, 1993). This indicates the functional importance of this region with respect to the regulation of *DEF* gene expression. For instance, a CArG-box motif present in this region of the *DEF* promoter (Figure 1a) was shown to be the binding site for the DEF and GLO heterodimer (Tröbner *et al.*, 1992). The CArG-box is not a unique feature of the DEF promoter, as it is
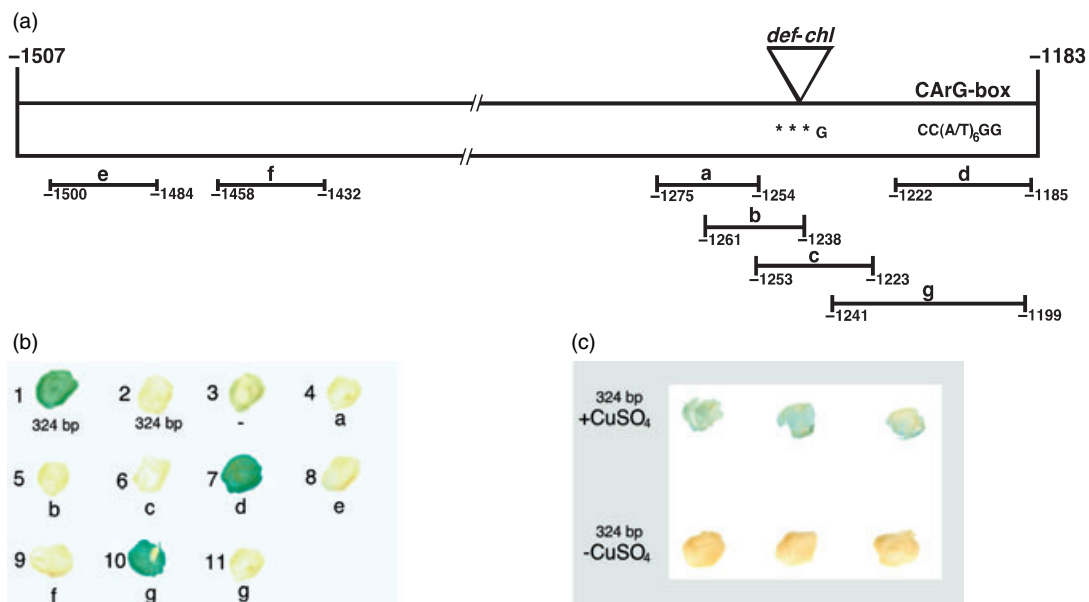

Figure 1. *DEF* promoter region used as bait (a) and β-Gal assay (b and c).

(a) Schematic representation of the 324 bp native *DEF* promoter region (open bar) used in the yeast one-hybrid screen. The sub-fragments used to generate the various yeast reporter strains (YRS) are indicated by letters (a–g). The numbers indicate the position of the various fragments with respect to the *DEF* transcription start. Except for the 324 bp fragment, all the other sub-fragments were present in the YRS as three tandem copies. The three base pair deletion (asterisks) and one base substitution (T → G) of the *def-chl* allele and the CArG-box are also indicated.

(b) β-Gal assay performed with the YRS (1–11) transformed with the chimerical *AD-BB15* construct. Most YRS carry the promoter fragments indicated together with *AD-BB15*, except for 2 and 11 that carry the 324 bp *DEF* promoter fragment and the sub-fragment 'g', respectively, but without the *AD-BB15* construct. The control YRS 3 contains only the construct *AD-BB15*, but none of the indicated fragments.

(c) β-Gal assay performed with *BB15* under the control of the inducible copper promoter but without the GAL4 activation domain in a yeast reporter strain containing the 324 bp *DEF* promoter fragment.

also found in promoters of other MADS-box genes (Tröbner *et al.*, 1992), and in promoters of genes involved in other aspects of floral development such as *MIXTA* (Noda *et al.*, 1994; C. Martin, John Innes Center, Norwich, UK, personal communication) and *DIVARICATA* (Galego and Almeida, 2002), although the functional relevance of this motif for the latter two genes has not been established yet.

Isolation of RSI *cDNA using the yeast one-hybrid system*

To isolate and characterize *trans*-acting factors capable of binding and regulating the activity of the *DEF* gene, we used the yeast one-hybrid technology (Li and Herskowitz, 1993; Wang and Reed, 1993). As bait for the one-hybrid screen we chose a 324 bp long fragment (between −1507 and −1183) of the *DEF* promoter containing the conserved region mentioned above. This DNA fragment had a low reporter gene expression in yeast. The fragment was further dissected into seven sub-fragments that were cloned in the yeast reporter vectors as three tandem repeats to later test the specificity of a putative DNA-binding protein isolated in the yeast one-hybrid screen (Figure 1). mRNA isolated from *A. majus* inflorescences carrying flowers at various developmental stages was used to construct a cDNA expression library that harbours in-frame the GAL4 activation domain (GAL4-AD). This library was used to transform the yeast reporter strain containing the 324 bp *DEF* promoter fragment.

About 10×10^6 yeast transformants were tested for reporter gene expression. About 300 candidates were isolated for their ability to grow in selective medium containing 15 mM 3-AT (see Experimental procedures). One candidate, containing a putative *trans*-acting factor named AD-BB15, was selected for further characterization because of its fast and specific response in the *LacZ* assay. Testing the ability of the *AD-BB15* gene product to activate the *LacZ* reporter gene in the yeast reporter strains (YRS) containing the various sub-fragments, revealed interaction with fragments containing the CArG-box. As strong reporter gene expression was observed with fragments 'd' and 'g', we concluded that the CArG-box plus 6 bp 5′ flanking and 7 bp 3′ flanking sequences were sufficient to drive reporter gene expression (Figure 1a). Quantification of β-Gal activity by liquid assay gave similar results (Figure S1). The activating function of AD-BB15 was independent of the yeast genetic background and vectors used, as similar results were also obtained with the yeast strain yWAM2 (Wolf *et al.*, 1996) in which, contrary to the yeast strain YM4271, the reporter constructs were maintained episomally (Figure S2). When the BB15 protein, without GAL4-AD, was expressed under the control of an inducible copper promoter in the yeast strain yWAM2 containing the 324 bp *DEF* promoter fragment, reporter gene expression was detected only in minimal medium supplemented with 100 μM $CuSO_4$ (Figure 1b). Hence, BB15 promoted *LacZ* expression without GAL4-AD, an indication

that BB15 contained an activation domain of its own. The partial *BB15* cDNA is 1676 bp long with the potential to encode a 507 amino acid peptide. Screening of an *A. majus* floral bud cDNA library (approximately 10^6 pfu) using the *BB15* cDNA as a probe led to the isolation of a 1890-bp long cDNA which appeared to contain the complete open reading frame (ORF) and 61 bp of the 5′ untranslated leader. The ORF encoded a 558-amino acid long protein. The corresponding gene was named *ROSINA* (*RSI*) (Figure 2).

Features of the RSI *gene product*

The RSI protein did not show a well-conserved amino acid sequence similarity to any gene product present in the database; rather RSI exhibited domains similar to known proteins from organisms of different phyla. For instance, among the proteins retrieved from the database with the C-terminal part of RSI, ATF6, a member of the mammalian b-ZIP transcription factor family was found (Hai *et al.*, 1989). The sequence similarity was restricted to part of the basic domain and to the b-ZIP domain of ATF6 (data not shown). The basic region of RSI, extending from amino acids 421 to 442, was followed by a 'zipper' domain consisting of three leucines and one isoleucine (Figure 2). Between amino acids 142 and 156, the repeated motif G-VYENDD-E-VYENDD-G was detected (Figure 2). Similar motifs represent sequence-specific sites for the binding of SH2-domain proteins. The tyrosine present in this RSI motif, once phosphorylated, could be a putative target of SH2 proteins (Cantley *et al.*, 1991; Songyang *et al.*, 1993). Based on its primary amino acid sequence, RSI appears to be a protein with domains similar to b-ZIP proteins. However, sequence similarity of RSI to proteins present in the database is too low, so that no phylogenetic relationships can be demonstrated.

The genomic status of RSI

Southern blot analysis revealed that *RSI* was a multi-copy gene with about 12–14 copies per genome, depending on which *A. majus* line was analysed (data not shown). Genomic sequencing data of several *RSI* copies showed sequence similarity at the nucleic acid level above 93%. In addition, most, if not all, *RSI* copies were located within the terminal inverted repeats (TIR) of a new transposable element of the CACTA family (unpublished data). For this reason, we favour the idea that *RSI* does not constitute a gene family *sensu strictu*, but is rather a gene repeated several times in the genome of *A. majus*, most likely due to transposition events. The CACTA transposable element carrying the *RSI* gene also encoded a gene product that showed a high amino acid sequence similarity to plant transposases (data not shown). This putative transposase gene was always found in opposite orientation with respect to *RSI* transcription, and did not overlap with the *RSI* sequence.

Figure 2. cDNA sequence and deduced amino acid sequence of *ROSINA* (*RSI*) (AJ 420968). A dash line underlines the RSI basic domain and the squares indicate the basic residues within this domain. The four zipper amino acids (L, L, L and I) are encircled. The repeated sequence motif is underlined. Open triangles indicate intron positions. Numbers to the right of the sequence denote the positions of nucleotide and amino acid residues respectively.

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GTGCATGGCTTGAAGAAACTTGACAATAGGGACAAAGAAGCTGTTAATTTCTCATAGAACCAGATGGATGCCAGAAATCCTATGACAGGC   90
                                                    M  D  A  R  N  P  M  T  G    9

AGAGGTGGACGTGCAACGAACATTGTTGCAAGCAGAGGCAGAGGTAGAGGTAGGAACCAGACGTCGTCTCGGGCACCCACGAGACCAGAT  180
R  G  G  R  A  T  N  I  V  A  S  R  G  R  G  R  G  R  N  Q  T  S  S  R  A  P  T  R  P  D   39

TCCCTGACAGTGGCTGGACTTCAGGGTAATATTGGAGGCACAATGCAGCCAGGTCCGTCAGTATCATATCCCGGTGGTATCCCGGTGTAT  270
S  L  T  V  A  G  L  Q  G  N  I  G  G  T  M  Q  P  G  P  S  V  S  Y  P  G  G  I  P  V  Y   69

CGGGTGTATCGTGGTGGTACATCTAGACATTTTACGCAGCCTGTTGCATCATCATCCCCTCGTTATTCAGCAGCACAGGTTCCCGTGCAT  360
R  V  Y  R  G  G  T  S  R  H  F  T  Q  P  V  A  S  S  S  P  R  Y  S  A  A  Q  V  P  V  H   99

CAGTCATTTGGTGTAGGGCACATCTCGAGGTCTTCATCACAGGGATCACATTCAGCATCAATGCCAGCGAATGCCTTGCATCAACATGGA  450
Q  S  F  G  V  G  H  I  S  R  S  S  S  Q  G  S  H  S  A  S  M  P  A  N  A  L  H  Q  H  G   129

GAAATCGAGGGTGGAGAAGATGATATGGAGGTTCAGGGGGTGTATGAGAACGACGATGAGGTGTATGAGAACGACGATGGATCACGAGGC  540
E  I  E  G  G  E  D  D  M  E  V  Q  G  V  Y  E  N  D  D  E  V  Y  E  N  D  D  G  S  R  G   159

CGTTCCAAAGGCCTTCAGCCCGTCGCCAATCCAACAGAGAGGCCTTGGATTTCATTCCTTCAGAGCGGGGGGTTTAGAGATTCGGGGACG  630
R  S  K  G  L  Q  P  V  A  N  P  T  E  R  P  W  I  S  F  L  Q  S  G  G  F  R  D  S  G  T   189

GTGCATAGCACAATTGCCACAAACCTGAGGACCATGTGCAATAAAGTTTACTTGACATACGGCAAGTGGTTGGATCGGGACAAAGAAGCG  720
V  H  S  T  I  A  T  N  L  R  T  M  C  N  K  V  Y  L  T  Y  G  K  W  L  D  R  D  K  E  A   219

TGTTGGGGAAGATTTAAGAATACTTTTCAATGGGAACCAGAGATGGAGATGCGTGTCCGTGCTCAATACAATAAGGTTGCTCGTTTACGA  810
C  W  G  R  F  K  N  T  F  Q  W  E  P  E  M  E  M  R  V  R  A  Q  Y  N  K  V  A  R  L  R   249

TTCACTGGTTCGGTGAATTGTGCCAAGAGGTGGTGGCAATCTTGTTGTAAGGACCCCGAGTACGCTAGGAATCACAAAACTTGGATACCC  900
F  T  G  S  V  N  C  A  K  R  W  W  Q  S  C  C  K  D  P  E  Y  A  R  N  H  K  T  W  I  P   279

GAAGATTTACTTCTCGGCTATATCAATTATTGGAAGTCCACTGAAGCAAAGGTTAAGTCACTTACTGCCTCACAGAACCGCGACAAAGGA  990
E  D  L  L  L  G  Y  I  N  Y  W  K  S  T  E  A  K  V  K  S  L  T  A  S  Q  N  R  D  K  G   309

GAGAAGGTTAACTACTTAGGCGGCTCCATTGGTATTGATGGCCATAGGGAAAAACTGGCCATAAAACTAAATGTCAAGCCGGAGGATGTC 1080
E  K  V  N  Y  L  G  G  S  I  G  I  D  G  H  R  E  K  L  A  I  K  L  N  V  K  P  E  D  V   339

CCCTTTCATTTGCTTTGGAGAGCAACTCATGTTAAGATATTGGATGATGGAACTGAGGTGTGGGCCAATCCGAGAGTCAAGCAGGCATAT 1170
P  F  H  L  L  W  R  A  T  H  V  K  I  L  D  D  G  T  E  V  W  A  N  P  R  V  K  Q  A  Y   369

GATGCATACATTGCTTATTGCCAAAGTAAGTGGGAATTTGAGAATGATTGGCCGATTTGGGATTTGGCGAAGTGGTTGGAGATTACTGGG 1260
D  A  Y  I  A  Y  C  Q  S  K  W  E  F  E  N  D  W  P  I  W  D  L  A  K  W  L  E  I  T  G   399

CCGATAAAGAAGAATAGGCTGCCTGGTTTGCCAGTGTGCGGTTATCCAGAAATGTTAGGAATCCAGGTCCGTAAAGAACCTAAGTATATT 1350
P  I  K  K  N  R  L  P  G  L  P  V  C  G  Y  P  E  M  L  G  I  Q  V  [R] [K] E  P  [K] Y  I   429

CATGTCGAATCGAGCAGTAGAAAACGTGATTACGAGAGCCAAATTAAGCACCTACAATCGGATAATCAGGAGTTGAAGAAGGATAATGAA 1440
[H] V  E  S  S  S  [R] [K] [R] D  Y  E  S  Q  I  K  H (L) Q  S  D  N  Q  E (L) K  K  D  N  E   459

GACTTGAAGACTTGGTTGGCAGCGATATCGCAGAAGGTGGGCATGCCTATCCCTGAACGGTTTCAGACATCGATCCCGGAGACCTGTAAT 1530
D (L) K  T  W  L  A  A (I) S  Q  K  V  G  M  P  I  P  E  R  F  Q  T  S  I  P  E  T  C  N   489

GTGCCGACGGAACCTCACTTTGACTCCACCCACCAGACGGCTGATCTCGGCACTAGGCAGGAGCATGACGACGTGTACACGCCAGGACCA 1620
V  P  T  E  P  H  F  D  S  T  H  Q  T  A  D  L  G  T  R  Q  E  H  D  D  V  Y  T  P  G  P   519

CCAGAAAACAACGTTCAACAACCGCAACAGCCACATGGTTCCCAGCTTCATCGTAATAGCCAAGCTGGCGGTATTGACGAGCATGTCGAT 1710
P  E  N  N  V  Q  Q  P  Q  Q  P  H  G  S  Q  L  H  R  N  S  Q  A  G  G  I  D  E  H  V  D   549

TTTGGAGCTTACGCGTTTTTGAGCCAGTGATAAATTTTTTGTGATGATGACGATTTAGTACTACTCGAACTGATAGTTCGAGTTTTTTGA 1800
F  G  A  Y  A  F  L  S  Q  *  *                                                            558

TGGTTTTAAGCAGCTATTAACTGTTTTTGTTGATATTTTTGCTAATGAATCGGACGTCATTTTGGTTATGGTGTAAGTTTATTTCATCTC 1890
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RSI binds in vitro to the 'extended' DEF CArG-box

As activation of the reporter gene in the yeast one-hybrid system can be mediated via protein–protein interaction of RSI with endogenous yeast DNA-binding proteins, electro mobility shift assays (EMSA) were used as an independent method to test the DNA-binding ability of RSI. A purified glutathione-*S*-transferase–RSI fusion protein (GST–RSI) was used in this assay with a 32-bp *DEF* promoter fragment containing the CArG-box motif *DEF* CArG-1 (Tröbner *et al.*, 1992). As shown in Figure 3(a), GST–RSI was able to 'shift' the *DEF* CArG-1 fragment, whereas GST alone failed to do so. A similar result was found with RSI expressed using rabbit reticulocyte lysate (Figure S3). Two RSI N-terminal truncations that retain the basic and the b-ZIP domains (RΔ1, from amino acid position 201 to 558, and RΔ2, from position 358 to 558) were tested in a DNA-binding assay. Both truncated proteins formed complexes with the *DEF* CArG-1 motif

with different mobility, corresponding to their different sizes (Figure 3b, lanes 2 and 3). For the GST-RΔ2 truncation, a competition experiment was performed adding increasing amounts of cold fragment. The decrease in band intensity observed correlated with increased amounts of competitor DNA (Figure 3c, lanes 4–7).

The DNA-binding assay clearly demonstrated that RSI is a genuine DNA-binding protein. RSI bound specifically to the *DEF* promoter region containing the CArG-box. The two N-terminal truncations and the competition experiments indicated that a DNA-binding domain is located in the C-terminal part of RSI.

Temporal and spatial expression of RSI during floral development

The *in vivo* (yeast) and the *in vitro* data suggest that RSI could function as a regulator of the B-function gene
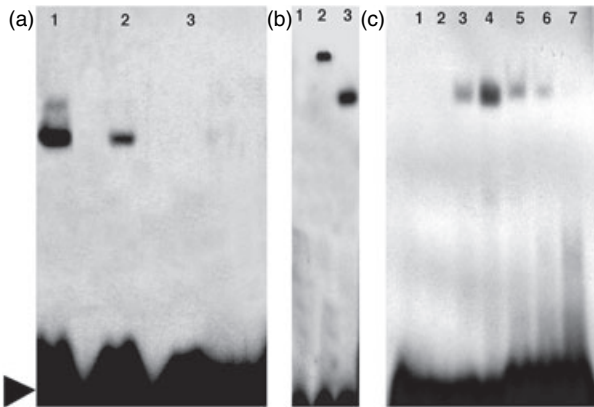
Figure 3. EMSA performed with the GST–RSI fusion protein (a), with two GST-N-terminal truncations (RΔ1 and RΔ2) (b) and in a competition experiment with the RΔ2 truncation (c). In all the (a), (b) and (c) lanes the *DEF* CArG-1 (Tröbner *et al.*, 1992) was used as probe.

(a) In lanes 1 and 2 the assay contained GST-RSI, in lane 3, GST protein only. In lane 2, 200-fold excess of poly-dIdC (0.2 μg) was used as unspecific probe competitor.

(b) Lane 1 contains free probe only, lanes 2 and 3, RΔ1 and RΔ2 protein respectively.

(c) Lane 1: free probe; lane 2: GST; lane 3: RΔ2 plus 500-fold excess of poly-dIdC (0.2 μg); lane 4: RΔ2 and lanes 5–7: 5×, 10× and 50× increasing amounts of cold probe respectively. An amount of 0.4 ng of [α-^{32}PdCTP]-labelled CArG-1 fragment was used in (c). The solid triangle indicates the position of free probe.

Figure 4. Level of RSI expression (a) and *in situ* localization of *RSI* transcripts (b).

(a) Quantitative RT-PCR performed on different wild-type plant tissues. The amplified products and the amount of single-strand cDNA used are indicated to the left and right of the autoradiography respectively.

(b) Longitudinal sections of wild-type *Antirrhinum majus* flowers from stage 6 onwards were hybridized with *DEF*-antisense RNA (1 and 2), *RSI*-antisense RNA (4–6), and with *RSI*-sense RNA (3). The signals appear as brownish-purple colour. The black arrows in 1 indicate a weaker signal of the *DEF* probe in the initial cells of sporogenous tissue compared with the connective tissues of the stamen (white arrows). The arrow in 4 indicates *RSI* localization in the initial cells of sporogenous tissue. The complementary expression patterns of *DEF* and *RSI* in the stamen are shown in 2 and 5. In carpels, *RSI* mRNA is detected in the stigma and in ovules (black arrows in 5 and 6). Note that a weak signal is detected in the inner layer of the ventral petal (white arrow in 5).
FB, floral buds; YS, young sepals; YP, young petals; YSt, young stamens; YC, young carpels; MS, mature sepals; MP, mature petals; MSt, mature stamens; MC, mature carpels; PA, primary apices; an, anther; c, carpel; ov, ovule; p, petal; s, sepal; st, stamen. Scale bar in (b) 100 μm, except for 3 where it is 200 μm.

DEFICIENS. To gain insight into the function of *RSI in planta*, its temporal and spatial expression was analysed. Northern blot experiments failed to detect the *RSI* transcript, either because only a few cells express it, or its steady-state level is too low. Therefore, quantitative RT-PCR analysis was performed with wild-type mRNAs from primary apices (PA), floral buds (FB) and from the four types of floral organs. The primers used in the RT-PCR experiment did not distinguish between the *RSI* transcripts produced by the various genomic *RSI* copies. RT-PCR directed to amplify a specific copy showed the same expression pattern, although the relative amount of a specific *RSI* transcript was reduced (data not shown). Therefore, we indicate with 'RSI transcript' the cumulative expression level of all expressed *RSI* copies. The *RSI* mRNA is found in PA and in FB (Figure 4a). In flowers, the *RSI* transcript was differentially expressed within the four types of floral organs. At a young stage of floral development (2–3 mm buds), the *RSI* mRNA was first detected in stamens. However, at later stages of flower growth (buds >10 mm), an increase in *RSI* transcript was observed in sepals and carpels. The amount of *RSI* transcript observed was quite low compared with that of *DEF* (Figure 4a), that was expressed at a similar level as previously reported by Northern analysis (Schwarz-Sommer *et al.*, 1992). These results showed that *RSI* is a developmentally regulated gene.
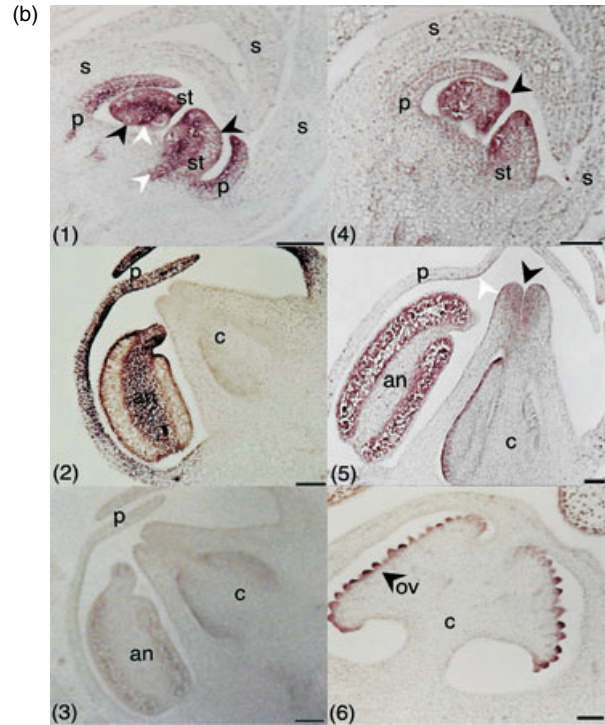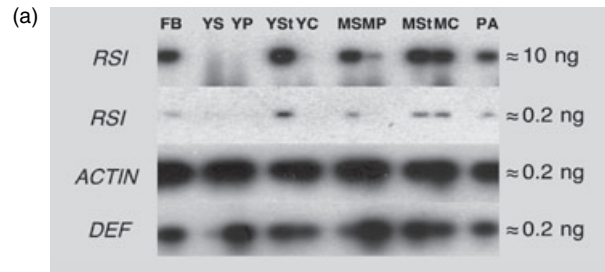
In situ *localization of* RSI *message*

RSI transcript accumulates in stamens earlier than in the other floral organs and might therefore overlap with the expression of the *DEF* gene. Alternatively, *RSI* could be

expressed in stamens within those cell types where *DEF* is not expressed. To clarify this aspect, *in situ* hybridization was performed on longitudinal sections of wild-type flowers using *RSI* and *DEF* anti-sense probes to compare their expression patterns. In this experiment, the *RSI in situ* probe was unable to distinguish between the various *RSI* transcripts, therefore the signal was considered as cumulative. *DEF* expression starts at stage 3 of floral development at the sites where petal and stamen primordia appear (Schwarz-Sommer *et al.*, 1992). In stamens from stage 6 onwards, the strongest *DEF* expression marked the incipient filaments and the connective tissue of the anthers (Figure 4, b1). At later floral developmental stages, *DEF* transcript was excluded from the sporogenous tissue (Figure 4, b2). In contrast, from stage 6 onwards, the *RSI* transcript was detected in a group of cells that give rise to the sporogenous tissue of the developing anthers (Figure 4, b4). At this developmental stage, the expression patterns of *RSI* and *DEF* appeared to be complementary. At later stages, the complementary expression patterns became more evident, with *RSI* being expressed in the tapetum and sporogenous tissue (Figure 4, compare b2 with b5). In petals, a signal was detected in the inner epidermal layer of the ventral petal at the position where this petal curves to form the lower lip (Figure 4, b5). The *in situ* experiment failed to detect *RSI* transcript in sepals. However, at later stages *RSI* mRNA was detected in the stigma and in ovules (Figure 4, b5 and b6). This expression study indicates that *RSI* is expressed within (ventral petals) and outside (tapetal cells and sporogenous tissue) the domain of expression of *DEF* as well as in tissues where normally *DEF* is not expressed (sepals and ovules), suggesting that it might have multiple functions in floral development, depending on the tissue where it is expressed. The complementary expression patterns of *RSI* and *DEF* observed within the stamens, together with the ability of RSI to bind to the CArG-box region of the *DEF* promoter, supports the hypothesis that RSI acts as a repressor of *DEF* activity in stamens. Hence, in an *rsi* loss-of-function mutant, an ectopic expression of *DEF* would be expected which could result in specific developmental alterations in at least three of the four floral organs.

Silencing RSI

The multi-copy nature of *RSI* and its possible redundancy rendered the isolation of *rsi* null mutants via reverse or forward genetics difficult. Therefore, the RNA interference (RNAi) strategy (Chuang and Meyerowitz, 2000; Hamilton and Baulcombe, 1999) was adopted with the aim of silencing all those copies expressing a functional *RSI* transcript. Although *A. majus* can be a recalcitrant species for transformation with *Agrobacterium tumefaciens*, protocols have been developed (Cui *et al.*, 2004; Heidmann *et al.*, 1998). Two parts of the *RSI* gene, *RNAi-1* of 137 bp (from 970 to 1108 bp) and *RNAi-2* of 245 bp (from 1268 to 1513 bp), respectively, were separately cloned in the pFGC1008 vector (see Experimental procedures) to promote dsRNA formation *in planta*. The constructs were used to transform the wild-type 165-E line. For both *RNAi-1* and *RNAi-2*, three independent shoots were regenerated and grown into mature plants. Two of three RNAi-1 transgenic lines, RNAi-1a and RNAi-1b, showed the same phenotypic alterations. Phenotypic alterations due to the dominant RNAi effect were observed in flowers of the T_1 generation plants and were transmitted to their T_2 progeny. These two transgenic lines featured single-copy T-DNA integration events and expressed the transgene (Figure S4). The three RNAi-2 transgenic lines did not show an obvious phenotype, and quantitative RT-PCR performed with one of these lines indicated similar expression levels of *RSI* and *DEF* as in the wild-type line (Figure S5). As RSI might regulate *DEF* expression we shall describe in more detail the phenotypes of the petals and stamens of the two RNAi-1 transgenic lines.

The phenotype of petals

The petals of the transgenic flowers displayed changes in shape and number. Moreover, both the outer and inner petal epidermis displayed outgrowths (ridges) characterized by alterations in cell morphology (Figure 5). The phenotypic changes in petal number and shape were seen in many, but not in all, flowers (>50%). In contrast, outgrowths, mainly restricted to the dorsal petal lobes, were detected in every flower. Frequently, an additional petal lobe developed between the two dorsal lobes of the transgenic flowers (Figure 5, a4 and a5). However, these altered flowers retained the characteristic dorso-ventral asymmetry of the wild-type *A. majus* flower. Changes in petal shape affected the ventral and lateral petals. The ventral petal acquired the shape and identity of a dorsal petal as seen in the *divaricata* mutant (Almeida *et al.*, 1997; Galego and Almeida, 2002), whereas the two lateral petals showed a reduced size, and both ventral and lateral petals were standing upright instead of having the characteristic curvature of wild-type petals. Often, in these dorsalized flowers, the ventral petals were duplicated becoming indistinguishable from the two dorsal petals (Figure 5, a7 and a8). This change had no detrimental effect on the two lateral petals that were retained. The corolla tube was not affected, except when dorsalization of the ventral petal occurred (Figure 5, a9). We never observed the converse: a ventralization of petals, as seen in the *cycloidea* mutant (Luo *et al.*, 1996). Moreover, an increase in localized outgrowths was observed in all flowers at the lower palate, where the two lateral and ventral petals fuse to form a hollow due to the outward curvature of these petals (Figure 5, a6). The inner epidermal layer of wild-type petal lobes is characterized by a unique landscape of conical cells, a light specialized type of cells (Noda *et al.*, 1994). In the two
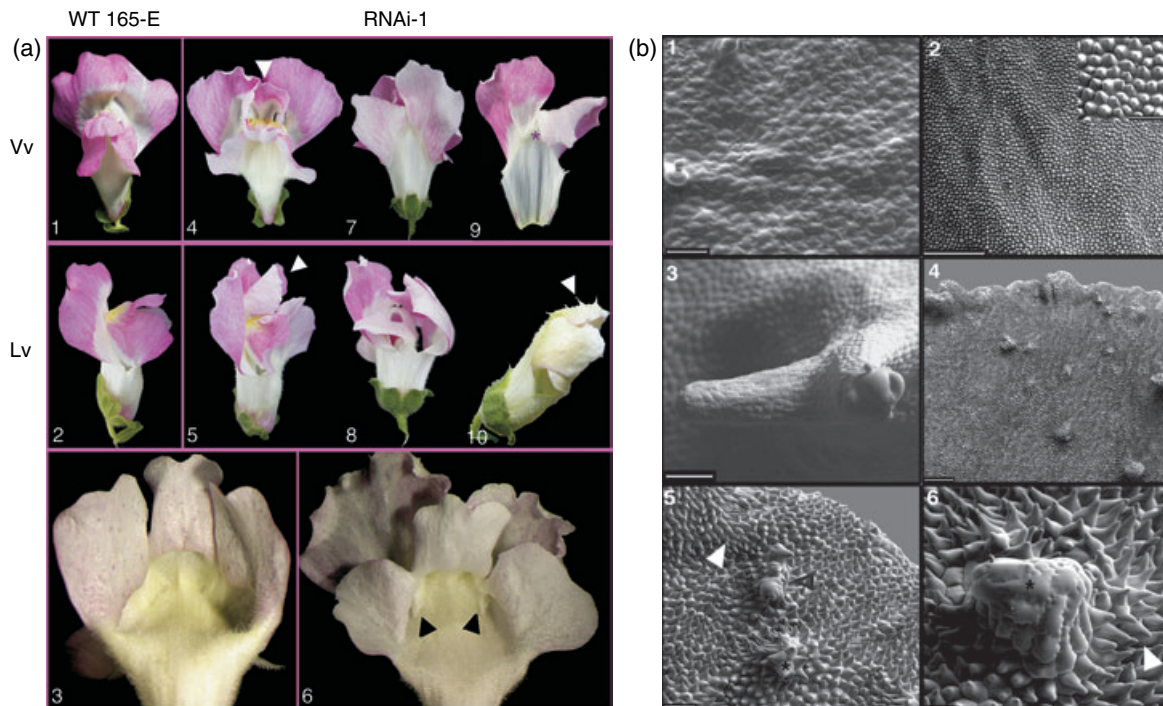
Figure 5. Flower phenotype (a) and SEM of the petal epidermis (b).

(a) Comparison of the flower phenotype of the wild-type 165-E (1–3) and of the RNAi-1 lines (4–10). V, ventral view; L, lateral view; 3 and 6, a close-up of the lower palate. The white triangle in 4 and 5 indicates an additional petal of an RNAi-1 transgenic flower that maintains its dorso-ventral symmetry. In 6, the two black triangles indicate the localized outgrowth in the lower palate. An RNAi-1 transgenic flower that has lost its dorso-ventral asymmetry is shown in 7 and 8. In 9, the inner side of the dorsalized ventral petal of the flower shown in 7 and 8 is revealed: the purple asterisk indicates the absence of the lower lip and of the two stripes of hairs within the corolla tube present in the wild-type flower (data not shown) that represent markers for dorso-ventral differences. In 10, the white triangle indicates outgrowths on the outer surface of an RNAi-1 dorsal petal lobe.

(b) SEM of wild type 165-E (1 and 2) and the RNAi-1 transgenic lines (3–6) of inner and outer epidermis of the dorsal petal lobes. In 3, outgrowth of the outer epidermis of the dorsal petal lobe of the RNAi-1 flower. Ridges disturb the homogeneous landscape of conical cells in the RNAi-1 lines (4–6). The shape of the RNAi-1 conical cells is more pointed [white triangle in 5 and 6 compared with wild-type conical cells (insert in 2, higher magnification)]. Asterisk in 5 indicates a rosette-like cell arrangement (see text). Amorphous cells are also shown (open triangle in 5). Outer epidermal cells cover an outgrowth in a conical cell landscape (asterisk in 6). Scale in (b) 5, 6 and insert in 2, 50 μm; 1, 100 μm; 3, 200 μm; 2 and 4, 500 μm.

transgenic lines, ridges of cells emerged apparently in a random fashion from the landscape of conical cells (Figure 5, b4). Some ridges were covered with conical cells, others with cells arranged in a rosette-like pattern mimicking a cell arrangement seen in the inner epidermis of the ventral petal lobe near the flower lip (Figure 5, b5). Other ridges were covered by cells having the morphology of the outer petal lobe epidermis, indicating a change in cell fate (Figure 5, b6). The petal lobe margin was also affected (Figure 5, b4).

The phenotype of stamens

Stamens did not show any morphological changes in their epidermal layer; however, the production of pollen was affected. Microspore development of the transgenic lines was delayed and tetrad separation was not completed. Although most of the surrounding callose matrix was degraded, rings of callose were still present at the point of contact of the tetrads or at one pole of the pollen cell. About 50% of the pollen grains were not viable and remained small (Figure 6).

All together, these phenotypic alterations suggest that *RSI* is involved in the regulation/modulation of *DEF* expression in petals and that it could act in establishing petal number and shape. However, the transgenic pollen phenotype did not directly correlate with the domain of *DEF* expression, although *DEF* transcript was slightly downregulated in the stamens (see later). One possible explanation of this phenotype is that a wild-type level of the *DEF* mRNA is necessary to establish the correct cell fate of the sporogenous tissue. Alternatively, RSI could control another gene/s involved in pollen maturation.

Occasionally, an increase in petal number was accompanied by an increase in sepal number with no epidermal cell alterations. The transgenic RNAi lines also displayed a mutant seed phenotype in accordance with *RSI* expression in ovules. As *RSI* expression in sepals and ovules is outside the domain of *DEF* expression, this aspect of *RSI* function will be described elsewhere (M. Roccaro, unpublished data).
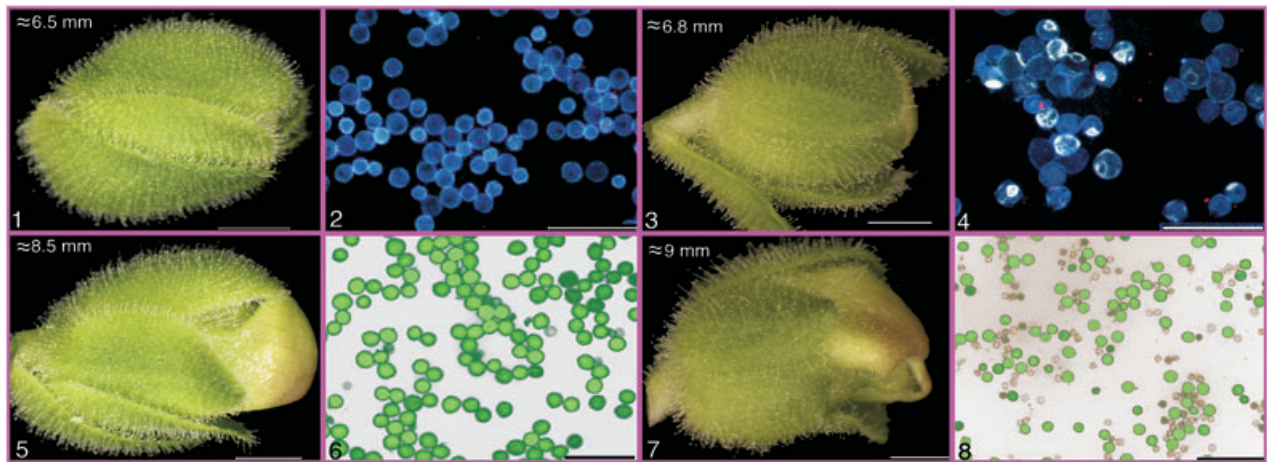
Figure 6. Pollen grain phenotypes of the RNAi-1 transgenic lines. Aniline staining and fluorescin diacetate (FDA) viability test of pollen grains for an unrelated RNAi transgenic line carrying the same vector with a different insert (2 and 6), and for the RNAi-1 transgenic line (4 and 8). Both transgenic lines have the 165-E genetic background. RNAi-1 pollen partially fails to degrade callose so that the tetrads do not separate. Rings of callose are visible as white signal at the pollen poles normally seen during pollen tube formation (4). FDA test reveals that 50% of the pollen grains die before full maturation (green pollen grains are viable; brownish pollen grains are not viable). Scale bar: 1, 3, 5 and 7, 2 mm; 2, 4, 6 and 8, 100 μm.

Nonetheless, the data presented here seem to indicate that not all phenotypic alterations observed can be attributed to RSI regulating *DEF*, but that the activity of *RSI* might have a broader functional spectrum.

Expression of RSI *in the RNAi-1 lines*

The aim of the RNAi experiment was to reduce or to abolish the amount of *RSI* transcript. However, to our surprise, we found an increased level of *RSI* transcripts in petals and stamens compared with the wild-type 165-E plants. During the characterization of the genomic structure of the RSI copies, we realized that in some copies fragments of the *RSI* sequence had been rearranged within the transposase, in opposite orientation (unpublished data). One of these copies was able to produce a transcript, *RSI-AT1* (*RSI-ABERRANT TRANSCRIPT1*), that had at its 3′-end a part of the 5′-end of *RSI* in anti-sense orientation, while its 5′-end belonged to the transposase (Figure 7). This could imply that in wild-type *A. majus* plants, double-stranded RNA (dsRNA) molecules form between the sense *RSI* transcript and this aberrant transcript leading to post-transcriptional control of *RSI* expression. In the RNAi transgenic lines, not *RSI* itself but rather the locus(i) able to produce aberrant *RSI* transcripts might be silenced in a strand-specific manner (Khvorova *et al.*, 2003; Parrish *et al.*, 2000; Schwarz *et al.*, 2003). Therefore, the silencing of *RSI*, via RNAi technology, in fact would result in an over-expression of *RSI*. We found by RT-PCR that the level of expression of *RSI-AT1* was reduced in petals and stamens supporting the above assumption (Figure 7). The *DEF* transcript was also monitored in the transgenic lines and compared with that of the wild type. The *DEF* tran-

script appeared slightly upregulated in petals, whereas in stamens the transcript was slightly reduced (Figure 7).

Phenotypes of 35S::RSI *transgenic* A. thaliana *plants*

Based on DNA and amino acid sequence analysis, the genome of *A. thaliana* does not seem to contain an *RSI* orthologue. Over-expression of *RSI* in this heterologous host might not have the same effect as in *A. majus*. To test this, *A. thaliana* plants were transformed with a *35S::RSI* construct. T_3 homozygous *35S::RSI* transgenic lines showed a phenotype surprisingly similar to the two RNAi-1 transgenic *A. majus* lines. The number, position and shape of first, second and third whorl organs was affected (Figure 8). The alterations persisted on average until 14–15 flowers were produced in the primary inflorescence. Later, flowers appeared to be normal. As we did not find any sequence identity between *RSI* and the *A. thaliana* genome to indicate a post-transcriptional effect, the phenotype seen in the *A. thaliana* transgenic lines could be attributed to the RSI protein that 'titrates out' factors normally involved in the specification of organ number, position and shape in this species. Alternatively, RSI could function as a direct regulator of the gene(s) involved in these developmental programmes. The similarity of phenotypes observed in *A. majus* and *A. thaliana* transgenic lines indicates that the recruitment of RSI is specific and suggests the existence of a functional *RSI* homologue in the *A. thaliana* genome.

Discussion

In *A. majus*, the expression patterns of *DEF* and *GLO* in petals and stamens clearly indicate that during development
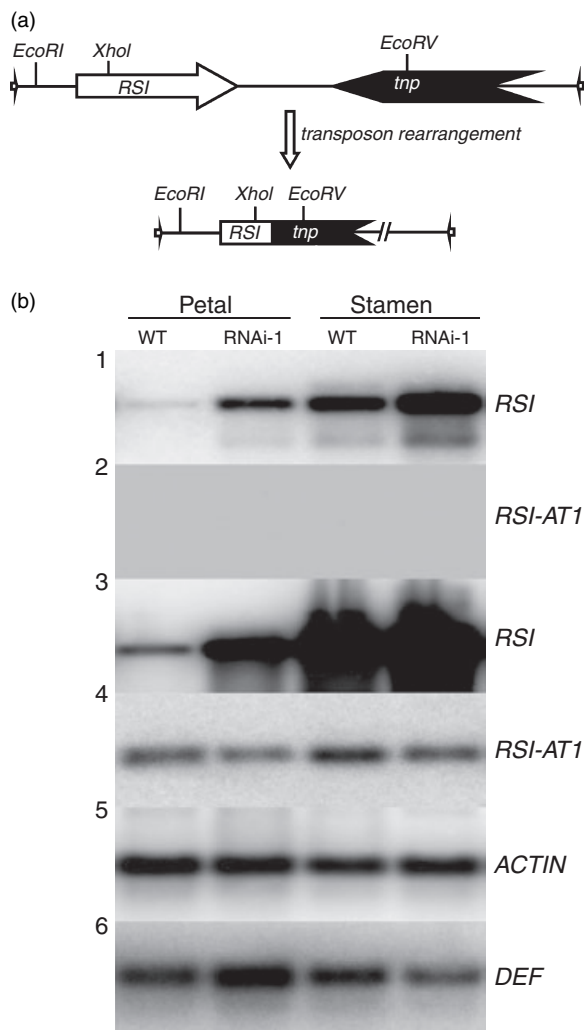
Figure 7. Schematic representation of the transposon-induced deletion that generates the fusion between the *RSI* and the transposase (tnp) genes (a), and comparison of *RSI* and *RSI-AT1* levels of expression in wild type and transgenic lines (b).

(a) *RSI* and tnp genes are not drawn in scale and without intron–exon structure. The small arrows symbolize the CACTA terminal inverted repeats. This rearranged transposon structure produces the *RSI-AT1* transcript.

(b) Quantitative RT-PCR performed on petals and stamens of the wild type 165-E and of the RNAi-1 transgenic lines using two rounds of amplification (see Experimental procedures). The amplified products are indicated on the right side of the panel. Note that the level of *RSI* expression is higher compared with that of the *RSI-AT1* (compare 1 with 2 in the first round amplification, or 3 with 4 in the second round amplification). Probes 1, 2, 3 and 4: *RSI*; 5: *ACTIN*; 6: *DEF*.
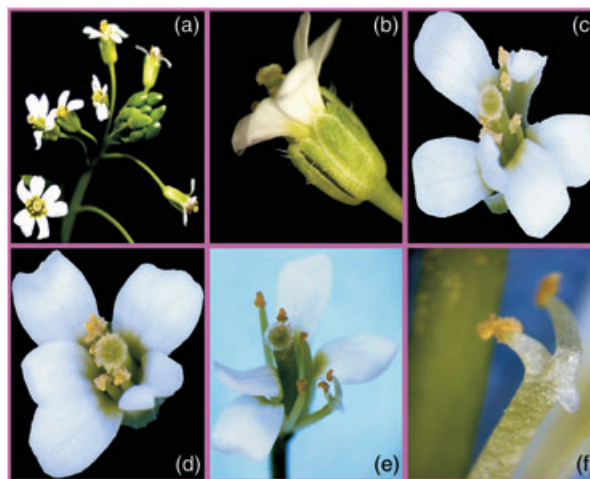

Figure 8. Phenotype of independent *Arabidopsis thaliana* transgenic lines harbouring a *35S::RSI* transgene. Number and shape of sepals, petals and stamens are affected in these transgenic lines.

(a) The main inflorescence of a *35S::RSI* line carries flowers with visible alterations in the number and position of the petals.

(b) A flower of a second *35S::RSI* line with increased number of sepals and petals; up to seven sepals were observed in this flower (only five are seen in this panel) in contrast to the four sepals present in the wild-type plant.

(c–e) Examples of mis-positioning and increased petal number and of mis-positioning and fusion of stamen of three independent *35S::RSI* transgenic lines.

(f) Close-up of the stamen fusion seen in (e). The phenotype of these lines is reminiscent of the *pan* phenotype (Running and Meyerowitz, 1996).

fourth whorl. Other genes, like *FIMBRIATA* (*FIM*), *CHORI-PETALA* (*CHO*) and *DESPENTEADO* (*DESP*), also control *DEF* and *GLO* expression (Ingram *et al.*, 1997; Simon *et al.*, 1994; Wilkinson *et al.*, 2000). So far, none of these gene products have been shown to act directly on the *DEF* promoter.

RSI: a novel factor that binds the extended CArG-box of the DEF promoter

A 324 bp DNA fragment of the *DEF* promoter containing a conserved region was used as bait to isolate the ROSINA protein (Figure 1a). Based on its primary amino acid sequence, RSI cannot be assigned to a specific gene family. The mosaic nature of the RSI protein is reflected by the presence of several functional domains common to proteins with DNA-binding ability. Perhaps one explanation of the protein domain structure of RSI resides in the gene's genomic organization. *RSI* is part of a CACTA transposable element (unpublished data). Therefore, it could have quickly evolved through deletion, inversion and/or non-homologous recombination, typical functions of transposons (Gray, 2000 and references therein), as is suggested by the identification of the *RSI-AT1* transcript.

The DNA sequence motif that RSI binds in yeast was narrowed down to a 23-bp long fragment containing the *DEF* CArG-box plus 6 bp 5′ flanking and 7 bp 3′ flanking

a negative regulator(s) is required to suppress their activity in specific tissues and cell types in these two floral organs and to restrict their expression to whorls 2 and 3. Several genes have been identified that are involved in the regulation of organ- and tissue-specific *DEF* expression.

Two MADS-box genes from *A. majus*, *PLENA* (*PLE*) (Bradley *et al.*, 1993) and *FARINELLI* (*FAR*) (Davies *et al.*, 1999) appear to negatively control *DEF* expression in the

sequences (Figure 1b). The precise binding motif of RSI remains to be determined. The presence of other putative DNA-binding sites (data not shown) within this segment of the *DEF* promoter as well as the occurrence of similar sites in other promoters suggest that other factors could contribute to the DNA-binding specificity of RSI in planta. However, an *in vitro* experiment indicates that RSI alone can bind to the *DEF* CArG-1 fragment (Figure 3).

Domains of RSI *expression*

During floral development, the steady-state level of the *RSI* mRNA is very low compared with *DEF* and it is developmentally regulated (Figure 4a). In stamens, *RSI* is expressed in a complementary fashion with respect to the *DEF* mRNA (Figure 4b). This indicates that RSI might serve as a negative regulator to suppress *DEF* transcription in the initial cells of the sporogenous tissue. Conversely, RSI could have a dual function, acting as a repressor or activator depending on the tissue type where it is expressed, on the topology of the promoter region of the regulated gene(s) used and on the presence of other protein partners as discussed above. TnpA, a regulator of the *En/Spm* transposon, represents an example of such a dual function (Grant *et al.*, 1990). Both activation and repression are also shown by the maize transcription factor VIVIPAROUS-1 (Hattori *et al.*, 1992; Hoecker *et al.*, 1995). *RSI* expression is not confined to stamens as it is also detected, by RT-PCR, in sepals, petals and ovules (Figure 4a). In petals *RSI* is expressed in the inner epidermal layer of the ventral petal where this petal curves to form the lower lip. Here, RSI could serve, once again, as a modulator of *DEF* activity that, in the ventral petal, shows a differential expression (Schwarz-Sommer *et al.*, 1992). This domain of *RSI* expression is in agreement with the epidermal cell phenotype observed in the RNAi-1 trangenic lines. In the transgenic lines, the conical cells of the dorsal petal lobes resemble the conical cells in the ventral petal lobes (Figure 5), perhaps as a consequence of the upregulation of the *RSI* transcript. In addition, phenotypic alterations in the transgenic tissues not expressing *DEF* (pollen and seed) suggest that RSI also interferes with the activity of other genes.

Silencing and upregulation of RSI

RNAi efficiently lowers the expression level of genes in a sequence-specific manner in plants (Chuang and Meyerowitz, 2000; Hamilton and Baulcombe, 1999), and in a sequence- and strand-specific manner in animals (Fire *et al.*, 1998; Schwarz *et al.*, 2003; Sijen *et al.*, 2001; Sui *et al.*, 2002; Zamore *et al.*, 2000). An internal part of the *RSI* coding region used to induce gene silencing via RNAi led to a strand-specific reduction of an aberrant *RSI* transcript, *RSI-AT1*, resulting in an upregulation of the *RSI* gene(s) (Figure 7). It appears that the strand-specific silencing effect of the RNAi-1 transgenic

lines partially removes a constraint on the *RSI* gene(s) exerted by the aberrant transcripts produced by the rearranged *RSI/* transposase and mediated, perhaps, by dsRNA formation. It has been postulated that RNAi has evolved as a defence mechanism against the 'deleterious' activity of transposable elements (Ketting *et al.*, 1999; Sijen and Plasterk, 2003; Tabara *et al.*, 1999). Therefore, the *RSI/*CACTA transposon system appears to have a dual function: one is to establish the proper amount of *RSI* transcript for its function during floral development, and the second is to repress transposition of the CACTA transposon copies that carry *RSI*.

The *RNAi-1* construct exhibited specificity for the RNA strand able to exert the silencing effect (sense strand). Schwarz *et al.* (2003) reported a phenomenon termed 'siRNA functional asymmetry' that accounts for which strand of the dsRNA will be used to exert the RNA interference. This could explain the strand specificity observed in the RNAi experiment.

RSI involvement in floral development

Within the expression domain of class B-function genes, RSI appears to regulate the number and shape of petals and to restrict the expression of *DEF* in stamens. In *A. thaliana* the b-ZIP factor *PERIANTHIA* (*PAN*) is involved in the determination of the number of floral organs within the three outermost floral whorls. Mutations in the *PAN* gene transform flowers from a tetramerous to a pentamerous stereotypical pattern (Running and Meyerowitz, 1996). Several functional aspects of the *RSI* gene are analogous to those of the *PAN* gene. For instance, both genes have a role in the establishment of the petal organ number and both genes contain a basic domain followed by a b-ZIP domain. They also have expression in the third and fourth whorls in common (Chuang *et al.*, 1999). The change in petal number in *A. thaliana* 35S::*RSI* transgenic lines is achieved by overexpression of *RSI* whereas the *pan* mutation represents a loss-of-function phenotype. Thus, the *A. thaliana* 35S::*RSI* phenotype could be explained by a 'titration effect' of RSI on factors such as PAN and/or its interacting partners.

Is RSI controlling *DEF* activity? No unambiguous answer can be given yet, as no complete null phenotypes of *RSI* are available. It is difficult to obtain such a mutant, as *RSI* is a multi-copy gene. However, the data presented here seem to support the idea that RSI can serve as a direct regulator of *DEF* activity.

Experimental procedures

Yeast reporter vectors, YRS and yeast one-hybrid screen

The native 324 bp *DEF* promoter fragment used in the one-hybrid screen, spanning the region from position −1507 to −1183, was prepared by PCR from wild type (line 165-E)

genomic DNA, using primers with appropriate restriction sites. Seven sub-fragments covering parts of the 324 bp sequence were chemically synthesized as complementary oligonucleotides and annealed to generate one, two or three tandem copies depending on the length of the fragment. The *pHISi-1* and *pLacZi* (Clontech, BD Biosciences Clontech, Heidelberg, Germany) reporter constructs were integrated in the genome of the yeast strain YM4271, complementing the mutated *HIS*3 and *URA*3 genes respectively. The yeast strains were transformed using the lithium acetate method (Gietz *et al.*, 1992) to generate YRS. All the YRS were tested for background levels of *HIS*3 reporter expression by adding increasing amounts of 3-amino-1,2,4-triazole (3-AT) (Sigma, Taufkirchen, Germany), an inhibitor of histidine (His) synthesis.

A cDNA expression library in *pGAD424* (Clontech) was made as previously described (Davies *et al.*, 1996). Library transformation and screening was performed as suggested by the Clontech One-Hybrid manual.

E. coli GST–RSI *fusion protein and DNA-binding assay*

The complete *RSI* coding sequence and two truncations of RSI (from amino acid positions 201 to 558, and from positions 358 to 558) were fused to the GST protein in *pGEX-5X-1* and named GST-RSI, RΔ1 and RΔ2 respectively. The resulting constructs were separately used to transform chemically competent *E. coli Bl21* (DE3) RIL cells (Stratagene, Amsterdam, the Netherlands). The GST–RSI proteins were purified using a microspin GST column (Amersham Bioscience, Freiburg, Germany), following the instruction of the manufacturer. The eluted protein was dialysed against 0.5x binding buffer [10 mM HEPES (pH 8), 10% glycerol, 50 mM KCl, 0.1 mM EDTA, 0.25 mM DTT] (Hai *et al.*, 1989).

The *DEF* CArG-1 DNA fragment used in the DNA-binding assay is identical to the one reported in Tröbner *et al.* (1992). The *DEF* CArG-1 DNA fragment was radioactively labelled by 'fill-in' with Klenow polymerase with [α-^{32}P]dCTP and purified from unincorporated radioactivity by acrylamide gel electrophoresis. The binding reaction (20 μl) contained 1 ng of DNA fragment, 0.2 μg of polydI-dC, and 10 μl of saturating levels of GST-RSI (100 ng μl^{-1}) in 0.5 x binding buffer. The binding reaction was carried out by incubation on ice for 30 min before loading on a 5% acrylamide:bisacrylamide (39:1) gel containing 0.5 x TBE.

RT-PCR analysis and in situ *hybridization*

First-strand cDNA was synthesized from mRNA primed with oligo-d(T)$_{15}$. The amount of single-stranded cDNA (ss-cDNA) that was produced was determined by measuring the amount of [α-^{32}P]dCTP that was incorporated. The specificity of the primer combination for *RSI* and *RSI-AT1* was checked on the available *RSI* cDNA and genomic clones, whereas for

DEF it was checked using single-strand cDNA made from *def* mutant plants. The primer combinations used for *RSI*, *DEF* and *ACTIN* yielded the complete coding regions after RT-PCR, while for *RSI-AT1* it yielded a product of 329 bp. The RT-PCR in Figure 7 was performed with two rounds of amplification. In the first round of amplification, 20 cycles were used for *RSI* and *RSI-AT1*, whereas 10 cycles were used for *ACTIN* and *DEF*. In the second round of amplification, 10 and 20 additional cycles were given to *RSI* and *RSI-AT1*, respectively, using 1 μl of sample from the first round amplification. Five microlitres per PCR reactions was loaded on a 0.7% agarose gel, separated by electrophoresis and alkaline blotted. The filter was hybridized with the corresponding probe at 70°C in 3x SSC for 6 h. The filter was washed twice with 0.1x SSC for 30 min. *In situ* hybridization was performed as described by Jackson (1991).

A. majus and A. thaliana *transformation*

Antirrhinum majus was transformed using the method developed by Heidmann *et al.* (1998), with minor modifications. The pFGC1008 vector was used to produce dsRNA as described in http://www.chromdb.org/. *Arabidopsis thaliana* (Columbia ecotype) was transformed using the floral dip method (Clough and Bent, 1998) with a derivative of pBIN19 (Bevan, 1984).

Microscopy

Aniline staining of pollen was performed following the method of Smith and McCully (1978). For the pollen viability test, anthers at different developmental stages were 'squeezed' with a forceps in the presence of few drops of 50 μg ml^{-1} of fluorescin diacetate, and after 5 min visualized under a UV light microscope. Scanning electromicroscopy was performed as described in Unte *et al.* (2003).

Supplementary Material

The following supplementary material is available for this article online:
Figure S1. β-Gal liquid assay performed with the YM4271 YRS (1–9) carrying AD-BB15 or the full-length AD-RSI.
Figure S2. β-Gal solid assay performed with yWAM2 YRS (1–9) carrying the *DEF* promoter fragments episomally.
Figure S3. EMSA performed with RSI alone or in combination with putative interacting partners expressed in rabbit reticulocyte lysate.
Figure S4. Southern hybridization to determine the transgene copy number (a), RT-PCR to detect the expression of the transgene (b) and schematic representation of RNAi-1 tandem inverted repeat (c).

Figure S5. RT-PCR to detect the expression of the RNAi-2 transgene in two of three RNAi-2 lines (a and b) and quantitative RT-PCR to determine the expression levels of *RSI* and *DEF* transcripts in wild type line, in the RNAi-2a and RNAi-1a transgenic lines respectively (c and d).

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