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FEATURED ARTICLE

A subcellular tug of war involving three MYB-like proteins underlies a molecular antagonism in *Antirrhinum* flower asymmetry

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SUMMARY

The establishment of meristematic domains with different transcriptional activity is essential for many developmental processes. The asymmetry of the *Antirrhinum majus* flower is established by transcription factors with an asymmetric pattern of activity. To understand how this asymmetrical pattern is established, we studied the molecular mechanism through which the dorsal MYB protein RADIALIS (RAD) restricts the activity of the MYB transcription factor DIVARICATA (DIV) to the ventral region of the flower meristem. We show that RAD competes with DIV for binding with other MYB-like proteins, termed DRIF1 and DRIF2 (DIV-and-RAD-interacting-factors). DRIF1 and DIV interact to form a protein complex that binds to the DIV-DNA consensus region, suggesting that the DRIFs act as co-regulators of DIV transcriptional activity. In the presence of RAD, the interaction between DRIF1 and DIV bound to DNA is disrupted. Moreover, the DRIFs are sequestered in the cytoplasm by RAD, thus, preventing or reducing the formation of DRIF-DIV heterodimers in the nuclei. Our results suggest that in the dorsal region of the *Antirrhinum* flower meristem the dorsal protein RAD antagonises the activity of the ventral identity protein DIV in a subcellular competition for a DRIF protein promoting the establishment of the asymmetric pattern of gene activity in the *Antirrhinum* flower.

Keywords: RADIALIS, DIVARICATA, molecular antagonism, myb-like proteins, *Antirrhinum majus*, dorso-ventral asymmetry.

INTRODUCTION

Many developmental patterns depend on the establishment of meristematic domains with different transcriptional activities, but the molecular mechanisms by which transcription factors interact to define distinct domains are often unclear. Dorsoventral asymmetry in *Antirrhinum majus* flowers requires the establishment of dorsal and ventral domains of gene activities through the combined action of transcription factors (Luo *et al.*, 1996, 1999; Galego and Almeida, 2002; Corley *et al.*, 2005). Such genetic interactions were characterised on the basis of changes in phenotypic or gene expression patterns caused by mutations, but the molecular and cellular mechanisms by which some of these transcription factors interact are poorly understood. To address this, we have examined the molecular interaction between RADIALIS (RAD) and DIVARICATA (DIV), two MYB proteins with contrasting effects on the dorsoventral pattern of *Antirrhinum* flowers.

Antirrhinum flowers are asymmetrical, with clear morphological differences between upper (dorsal) and lower (lateral and ventral) petals. This asymmetry depends on the activities of two dorsally expressed genes, *CYCLOIDEA* (*CYC*) and *DICHOTOMA* (*DICH*), which encode members of

the TCP family of transcription factors (Luo *et al.*, 1996, 1999; Cubas *et al.*, 1999). *CYC* and *DICH* act in a partially redundant manner to positively regulate *RAD*, a gene encoding a MYB protein also dorsally expressed (Corley *et al.*, 2005). Double *cyc;dich* or single *rad* mutant plants produce flowers in which dorsal identity is lost. In these mutants, ventral identity spreads to dorsal positions and all the petals assume a ventral identity, suggesting that the role of dorsal-acting genes might be to inhibit the activity of ventral-acting genes. *RAD* acts downstream of *CYC* and *DICH* (Corley *et al.*, 2005; Costa *et al.*, 2005) and it is likely to be the dorsal factor antagonising ventral-acting genes. One such gene, *DIV*, encodes a MYB protein required for specifying ventral identity. A mutation in *DIV* causes the loss of the ventral identity of the flower (Almeida *et al.*, 1997; Galego and Almeida, 2002). Furthermore, in a *cyc; dich;div* mutant the ventralising effect in the dorsal part of the flower observed in *cyc;dich* mutants is also lost (Almeida *et al.*, 1997), indicating that, in a *cyc;dich* background, *DIV* confers ventral identity to all positions around the flower. Additionally, plants that ectopically express *RAD* produce flowers without ventral identity that resemble the *div* mutant (Cui *et al.*, 2010). This is consistent with the idea that the dorsal gene *RAD* is antagonising the activity of the ventral identity gene *DIV*. The molecular and cellular basis for such antagonism is, however, poorly understood.

Although the phenotypical effect of *DIV* inactivation is restricted to ventral regions of the corolla, the gene is transcribed in all parts of the flower (Almeida *et al.*, 1997; Galego and Almeida, 2002). This suggests that the antagonistic effect of *RAD* over *DIV* is not exerted at the transcriptional level. A clue to how dorsal- and ventral-acting genes might interact comes from analysing the amino acid sequences of the RAD and DIV proteins. *DIV* encodes for a 308 amino acid protein with two MYB domains, the C-terminal one being very similar to domains involved in DNA binding in other DIV-related MYB proteins, indicating that DIV may act as a transcription factor (Rose *et al.*, 1999; Galego and Almeida, 2002). The N-terminal MYB domain is thought to be involved in protein–protein interactions rather than in DNA binding (Rose *et al.*, 1999). RAD, in turn, is a small protein with 93 amino acids and a single MYB domain that is 52% identical to the N-terminal MYB domain of DIV (Corley *et al.*, 2005). Thus, rather than acting as a DNA-binding transcription factor, RAD is likely to act through a mechanism involving protein–protein interactions. A precedent for a possible mode of interaction between RAD and DIV comes from the finding of small proteins, known as small interfering peptides (siPEP), which contain a protein–protein interaction domain similar to that of the antagonised transcription factor; most lack a DNA-binding domain (Seo *et al.*, 2011). Most transcription factors bind DNA and activate transcription in the form of homo- or heterodimers. The siPEPs can disrupt the formation of these dimers by competitively forming non-functional heterodimers that are incapable of activating transcription (Benezra *et al.*, 1990; Reed, 2001). This seems to be the case in tomato where a RAD homologue (FSM1) is thought to negatively regulate cell expansion of the fruit by competing with a DIV homologue (MYBI) for FSB1, another MYB protein (Machemer *et al.*, 2011). However, accumulating evidence supports that transcriptional antagonisms have, in fact, broader molecular modes of action than just the formation of non-functional dimers. Heterodimerisation of siPEPs can sequester target transcription factors in the cytoplasm (Sasaki *et al.*, 2004) and heterodimers containing siPEPs that have DNA-binding domains can even compete with the transcription factor for the same DNA-binding domain (Chang *et al.*, 2003).

In the present study, we show that RAD and DIV interact neither directly by forming heterodimers nor by competing for DNA, rather they compete for common protein targets termed DRIFs (DIV-and-RAD-interacting-factors). DIV and DRIFs, themselves MYB-like proteins, have overlapping expression patterns and can form heterodimer complexes that bind to DNA containing a DIV consensus sequence. RAD prevents these heterodimers from being established either by binding directly to a DRIF protein in the nucleus or/and by sequestering the DRIF proteins in the cytoplasm. We therefore propose that RAD antagonises DIV in subcellular competition for a DRIF protein by inhibiting the interaction between DIV and DRIFs in the dorsal regions of the *Antirrhinum* flower in order to establish the asymmetric pattern of gene activity in the flower meristem.

RESULTS

DIV is not directly antagonised by RAD

The antagonism that RAD exerts over DIV in the establishment of the dorsal identity of the *Antirrhinum* flower most likely reflects a molecular competitive inhibition of RAD over DIV transcriptional activity. To understand the nature of this molecular mechanism, we tested whether RAD and DIV could be competing for the same DNA target sequence by electrophoretic mobility shift assay (EMSA). Therefore, the optimal DNA-binding site of DIV recombinant protein produced and purified from *Escherichia coli* was determined on a binding-site selection assay using random oligonucleotides. All of the 34 DNA sequences obtained after five rounds of selection contained a consensus sequence homologous to an I-box binding site (Figure 1a). If the two existing MYB domains in the DIV protein bound to different DNA motifs, one would expect to obtain two different consensus sequences in this experiment. A single consensus sequence was, however, obtained, suggesting that DIV only binds to the determined consensus sequence. A DIV homologue in tomato was found to bind to a similar consensus DNA-binding site (Rose *et al.*, 1999). The DIV DNA-target sequence obtained on the random bind-

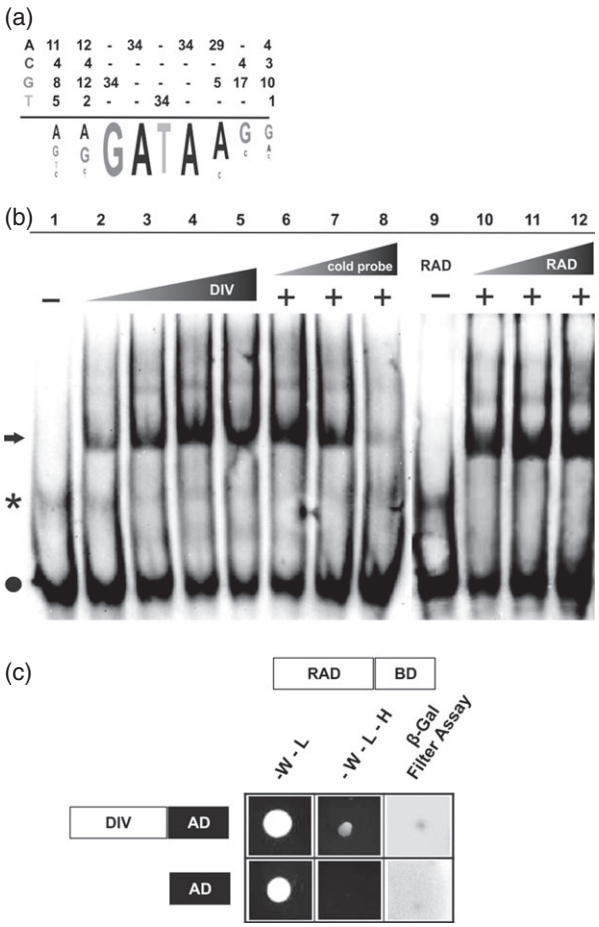

Figure 1. RAD does not interact with the DIV DNA-binding site nor with DIV protein.
(a) Consensus DNA-binding sequence for DIV protein obtained by random binding-site selection. After five rounds of selection, a total of 34 binding sites of DIV were piled up using MEME (http://meme.nbcr.net/meme/) and a consensus sequence was generated.
(b) DIV protein binding specificity to digoxigenin (DIG)-labelled probe (10 ng) containing the consensus region identified in (a) in the presence and absence of RAD protein: lane 1, no protein; lanes 2–5, 10, 50, 100 and 200 ng of DIV protein, respectively; lanes 6–8, DIV protein (100 ng) with 10, 100 ng and 1 μg of unlabelled probe, respectively; lane 9, RAD protein (1 μg); lanes 10–12, DIV protein (100 ng) with 10, 100 ng and 1 μg of RAD protein, respectively. The DIV–DNA complex is identified by an arrow, a background shift by an asterisk and the free probe by a full circle.
(c) RAD fused to the GAL4-binding domain (BD) was unable to activate the reporter genes (*HIS3* and *LacZ*) in cells co-transformed with DIV fused to the GAL4-activation domain (AD) in a yeast-two hybrid assay. (-W-L, medium lacking tryptophan and leucine; -W-L-H, medium lacking tryptophan, leucine and histidine.)

ing-site selection was used to synthesise a digoxigenin (DIG)-labelled DNA probe utilised in EMSAs. When this DNA probe was incubated with increasing amounts of DIV protein, a dose-dependent shift corresponding to the DIV–DNA complex was observed, confirming that DIV binds to the previously identified DNA-target sequence (Figure 1b). This DIV–DNA complex is specific because it is disturbed by the addition of increasing amounts of unlabelled DNA probe (cold probe). To test if RAD could bind to the same DNA-target sequence as DIV, RAD recombinant protein was mixed with DIV-target DNA. RAD did not bind to this DNA sequence (Figure 1b, lane 9), indicating that RAD most probably does not compete with DIV for the same DNA-target sequence.

Although RAD does not compete directly for the DIV DNA-binding site it could still prevent DIV from binding to its target DNA through a direct interaction with the DIV protein. This was tested by adding increasing amounts of RAD protein to the binding reaction containing DIV–DNA complex. The shift corresponding to the DIV–DNA complex was unaffected, showing that RAD is unable to directly disturb the interaction between DIV and its target DNA (Figure 1b). To test further whether RAD interacts directly with DIV, the open reading frame (ORF) of *RAD* was fused to the GAL4 DNA-binding domain (RAD-GAL4^{BD}) and the ORF of *DIV* fused to the GAL4 activation domain (DIV-GAL4^{AD}). Following transformation of yeast cells with both constructs, no activation of the reporter genes *LacZ* or *HIS3* on a yeast-two hybrid (Y2H) assay was observed, indicating that RAD and DIV do not interact directly with one another (Figure 1c).

These results suggest that DIV function is not antagonised directly by RAD through competition for the same DNA-target region nor through prevention of DIV from binding to its DNA target by the formation of RAD–DIV heterodimers.

RAD and DIV interact with a third MYB-like protein

An alternative scenario that could explain how RAD antagonises the function of DIV in the dorsal part of the flower primordia would be if RAD and DIV competed for a third protein that could determine the regulation of DIV targets via heterodimerisation with DIV. This being the case, the third protein would interact with both RAD and DIV. To identify RAD protein interactors, a Y2H cDNA library from *A. majus* flowers was screened using the RAD protein as bait (RAD-GAL4^{BD}). DIV could not be used as bait because it is a transcriptional activator in yeast (Figure 2c). Two similar MYB-like proteins, named DRIF1 and DRIF2, were the only proteins identified in the screening (Figure 2a), indicating that RAD has very few interactors.

For the DRIFs to be acting as mediators in the antagonism that RAD has over DIV, they should also have affinity for the DIV protein. This was tested in a Y2H assay using the fusions DRIFs-GAL4^{BD} and DIV-GAL4^{AD}, which showed that the DRIF proteins also interact with DIV (Figure 2b). As judged from the levels of β-galactosidase activity produced as a result of protein interactions, DRIFs can establish strong associations with both RAD and DIV (Figure 2c). A control Y2H assay using DRIFs-GAL4^{AD} and DRIFs-GAL4^{BD}, showed that the DRIFs could not form homo- or heterodimers (Figure 2c).

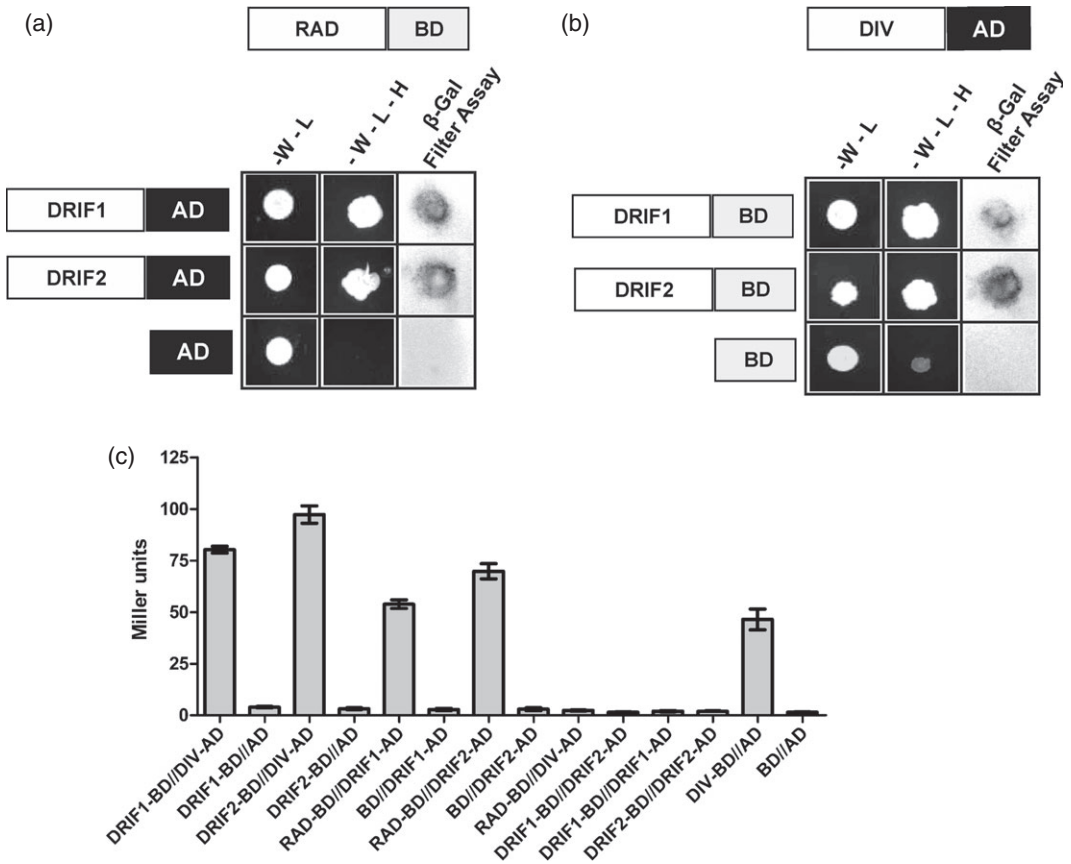

Figure 2. RAD and DIV interact with the same two MYB-like proteins.
(a) DRIF1 and DRIF2 were identified on a yeast two-hybrid (Y2H) screening of an *Antirrhinum* cDNA floral library using RAD as bait by the activation of the reporter genes *HIS3* and *LacZ*.
(b) DIV interacts with DRIF1 and DRIF2. DIV-GAL4^{AD} was able to activate the reporter genes when co-expressed with the DRIFs-GAL4^{BD}.
(c) Quantitative β-galactosidase liquid assay showing that DIV and RAD strongly interact with DRIF1 and DRIF2. The assay also shows that DRIF1 and DRIF2 do not homo- and heterodimerise and that DIV is able to activate transcription. BD/AD (negative control, representing, respectively, empty vectors pGBT9 and pGAD424). Values represent means of three replicas from three independent transformations (scale bar represents SD) and are expressed as Miller units. (-W-L, medium lacking tryptophan and leucine; -W-L-H, medium lacking tryptophan, leucine and histidine.)

In tomato (*Solanum lycopersicum*), RAD and DIV homologous proteins also interact with a DRIF-like protein (SlFSB1) involved in the regulation of cell expansion in the fruit, suggesting that the DRIF proteins are present in other organisms to regulate different developmental processes (Machemer *et al.*, 2011). The DRIFs belong to a small MYB-like family of proteins that contains two conserved domains. The first is an atypical MYB-like domain in which, one of the three regularly spaced tryptophan residues that characterise a MYB domain is replaced by a tyrosine (-W-X_{20-23}-Y-X_{20}-W-) (Figure S1). The second conserved domain has an unknown function and has been annotated as DUF3755.

To understand the evolutionary history of the DRIF protein family, a phylogenetic tree was built using the sequences of the two conserved domains (Data S1) in a broad range of plant species from mosses to angiosperms (Figure 3). The phylogeny shows that DRIFs are members of an ancient protein family with several homologues in all species of plants analysed, including *Physcomitrella patens*. Within the angiosperm lineages, the DRIF family can be found in both monocots and dicots, and is subdivided in two main clades designated as Group 1 and Group 2. Group 1 contains DRIF1 and DRIF2 from *Antirrhinum*, along with their closest homologues from the other species considered. Group 2 contains the other angiosperm DRIF homologues and particularly some that resulted from duplications after the separation between monocots and dicots. This can be observed in the clade that includes only sequences from the monocots *Brachypodium distachyon* and *Oryza sativa*. Group 2 also contains other homologues from *Arabidopsis thaliana*, *Lotus japonicus* and tomato, including SlFSB1, described previously in tomato as an interactor of RAD and DIV homologues (Machemer *et al.*, 2011).

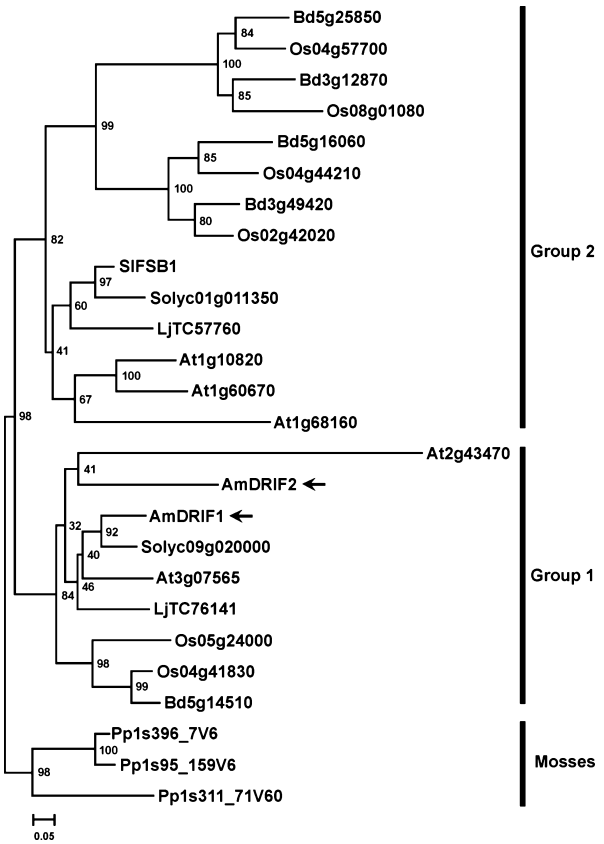

Figure 3. Phylogenetic tree representing the evolutionary history of the DRIF protein family.

The tree was constructed by analysing the conserved regions (MYB-like and extended DUF3755 domains) of the DRIF-like proteins with the neighbor-joining method. Percentage bootstrap values are indicated to the right of each node. Am, *Antirrhinum majus* (DRIF1, JX966358; DRIF2, JX966359); At, *Arabidopsis thaliana* (At3g07565; At2g43470; At1g10820; At1g60670; At1g68160); Bd, *Brachypodium distachyon* (Bd5g14510; Bd3g49420; Bd5g16060; Bd3g12870; Bd5g25850); Lj, *Lotus japonicus* (LjTC76141; LjTC76141; sequences originated from the DFCI lotus gene index); Os, *Oryza sativa* (Os05g24000; Os02g42020; Os04g44210; Os04g57700; Os08g01080; Os04g41830); Pp, *Physcomitrella patens* (Pp1s396_7V6; Pp1s95_159V6; Pp1s311_71V6; sequences originated from Joint Genome Institute); Sl, *Solanum lycopersicum* (SlFSB1, Solyc01g011350; Solyc09g020000; Solyc01g011350; sequences originated from the Sol Genomics Network). Arrows indicate DRIF1 and DRIF2 proteins from *Antirrhinum*. Scale bar: 0.05 indicates the evolutionary distance between the groups.

RAD disrupts the DRIF-DIV-DNA complex

To understand how the DRIF proteins might be mediating the antagonism that RAD exerts over DIV in the establishment of the asymmetric pattern of *Antirrhinum* flowers, the molecular mechanism through which the function of DIV might be disrupted need to be elucidated. One possibility could be that the DRIF proteins form heterodimers with DIV and participate in protein–DNA transcription complexes. RAD could then be antagonising DIV activity by disrupting the formation of such complexes. To test this hypothesis, binding of DIV and DRIF to the target sequence of DIV in the presence and absence of RAD was assayed by EMSA (Figure 4).

To perform this EMSA, the DRIF1 protein was expressed in *E. coli* in fusion with a glutathione-*S*-transferase (GST) tag. DRIF2 protein was not used in this assay due to its low levels of solubility in *E. coli*. To confirm that the GST-DRIF1 protein was functional, as well as to validate the interaction obtained in yeast, pull-down assays were performed with recombinant GST-DRIF1 protein and with the fusion proteins ECFP-RAD and EYFP-DIV expressed in *Nicotiana benthamiana.* GST-DRIF1 was able to pull-down both EYFP-DIV and ECFP-RAD, showing that these proteins are able to interact *in vitro* and indicating that the recombinant protein GST-DRIF1 was functional (Figure S2).

To test whether DRIF1 and DIV could heterodimerise and bind to DNA, increasing amounts of DRIF1 protein were added to the reaction containing DIV protein and its DNA probe. This reaction resulted in a gel shift corresponding to a complex with lower mobility than the complex corresponding to DIV bound to the probe (Figure 4, lanes 3–8). The DRIF1 protein on its own could not bind to the DIV DNA-binding site (Figure 4, lane 17). These results show that DRIF1 can form a super-complex with DIV bound to its DNA-target sequence.

To test if RAD could disrupt the DRIF1-DIV-DNA super-complex, increasing amounts of RAD protein were added in the reactions containing DRIF and DIV and the target DNA. In the presence of RAD, the low mobility shift disappears and the shift corresponding to DIV bound to DNA is re-established (Figure 4, lanes 10–16). This result indicates that RAD disrupts the formation of the DRIF1-DIV-DNA super-complex by competing *in vitro* for DRIF1.

RAD sequesters the DRIF proteins in the cytoplasm

To further investigate the molecular competition between RAD and DIV for the DRIF proteins and determine in which subcellular location these interact, the cytolocalisation of the proteins was analysed. *Nicotiana benthamiana* leaves were agro-infiltrated with constructs harbouring the ORFs of *DRIF*s, *RAD* and *DIV* fused to the fluorescent proteins *RFP*, *ECFP* and *EYFP*, respectively. The results showed that RAD is localised both in the cytoplasm and in the nucleus, including the nucleolus (Figure 5a). DIV is only observed in the nucleus and not in the nucleolus (Figure 5b). In contrast to DIV, DRIF1 is present in the cytoplasm and, in particular, surrounding the nucleus (Figure 5c). This spatial separation of DRIF1 and DIV in the cell would not allow them to interact *in vivo*. However, the two proteins were expressed in independent cells. Therefore, to test whether the cytolocalisation patterns of DRIF1 and DIV change in the presence of each other, these proteins were co-expressed. DIV localisation remained confined to the nucleus when co-expressed with *DRIF1* (Figure 5d). However, in the presence of DIV, the localisation of DRIF1 shifted from the cytoplasm to the nucleus. Thus, the DIV and DRIF1

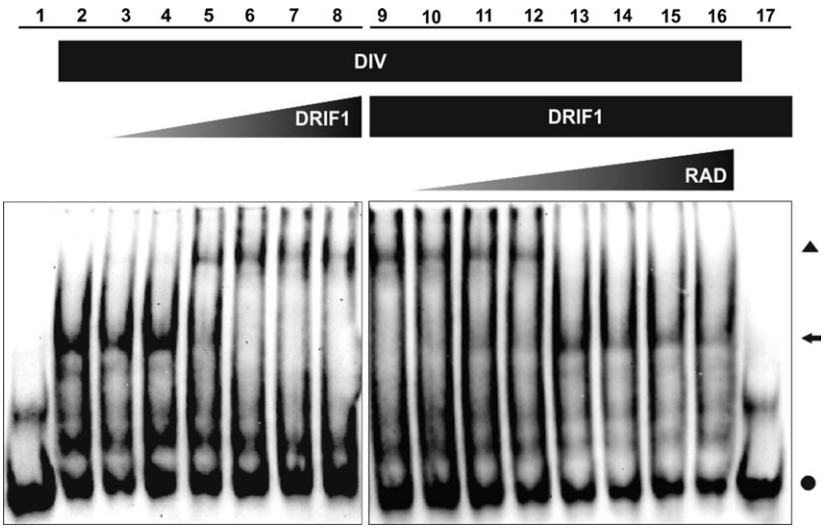

Figure 4. RAD disrupts the DRIF-DIV-DNA binding complex.
Electrophoretic mobility shift assay (EMSA) using 10 ng of a digoxigenin (DIG)-labelled probe containing DIV DNA-binding consensus: lane 1, free probe; lane 2, DIV protein (100 ng); lanes 3–8, DIV protein (100 ng) with 5, 8, 10, 15, 20 and 100 ng of DRIF1 protein, respectively; lane 9, DIV protein (100 ng) with DRIF1 protein (100 ng); lanes 10–16, DIV protein (100 ng) with DRIF1 protein (100 ng) and 0.01, 0.1, 0.5, 1, 10, 100 and 200 ng of RAD protein, respectively; lane 17, DRIF1 protein (100 ng). The DIV-DNA complex is identified by an arrow, the supershift corresponding to the DRIF1-DIV-DNA complex by a full triangle and the free probe by a full circle.

subcellular localisation patterns coincide (nuclear), as required if the two proteins are to interact *in vivo*. Further evidence that DIV and DRIF1 interact in the nucleus *in vivo*, came from a bimolecular fluorescence complementation (BiFC) experiment in which YFP_C-DRIF1 and YFP_N-DIV-YFP were co-expressed in tobacco leaves (Figure 5e). Yellow fluorescent protein (YFP) fluorescence was observed in the nuclei of transformed cells as the result of direct interaction between DRIF1 and DIV.

RAD localises to both cytoplasm and nucleus, and therefore might compete with DIV for DRIF1 in either or both cell compartments. To investigate this, the three fusion proteins were co-expressed and their subcellular localisations were determined. Whilst the localisations of RAD and DIV remain unaltered, in the presence of both DIV and RAD the DRIF1 protein localises only in the cytoplasm (Figure 5f), suggesting that RAD sequesters DRIF1 in the cytoplasm, thus possibly reducing the availability of DRIF1 to participate in a nuclear complex with DIV. Additionally, co-expression of only DRIF1 and RAD does not alter the cytoplasmic localisation of DRIF1 (Figure 5g). A BiFC experiment performed with YFPc-DRIF1 and YFPn-RAD confirmed that DRIF1 and RAD interact *in vivo* in the cytoplasm (Figure 5h). These results suggest that by interacting with RAD, DRIF1 is sequestered in the cytoplasm and, consequently, prevented from interacting with DIV in the nucleus.

Controls for the cytolocalisation performed by agroinfiltration show that the change of cytolocalisation of the DRIF1 protein is caused by an interaction with RAD or DIV and not by interactions between the fluorescent proteins (Figure S3). Confirmation of the molecular sizes of the fluorescent-tagged proteins is present in Figure S4. Controls for the BiFC experiment are also shown in Figure S3, including the expression of YFPc-DIV and YFPn-RAD that are both co-localised in the nucleus, therefore confirming that RAD and DIV do not interact *in planta*.

To determine whether DRIF2 had cytolocalisation dynamics similarly to DRIF1 when co-expressed with RAD and DIV, the same experiments as above were performed with DRIF2. The cytolocalisation pattern of DRIF2 differs from that of DRIF1. DRIF2 is only localised in the nucleus with a clear localisation in the nucleolus (Figure 5i). However, when co-expressed with DIV, DRIF2 is only observed inside the nucleus, but excluded from the nucleolus (Figure 5j). This shows that, similarly to DRIF1, DRIF2 cytolocalisation changes in the presence of DIV to the same subcellular localisation as DIV and suggests that DRIF2 is also able to form a nuclear complex with DIV. A BiFC experiment performed with YFPc-DRIF2 and YFPn-DIV confirmed that DRIF2 and DIV interact in the nucleus but outside the nucleolus (Figure 5k).

To test if RAD could also disturb the DRIF2-DIV nuclear complex, RAD was co-expressed with DRIF2 and DIV. In the presence of RAD and DIV, the DRIF2 protein is observed in the cytoplasm and the nucleus (Figure 5l), indicating that although some DRIF2 remains in the nucleus, RAD is able to sequester some DRIF2 protein in the cytoplasm. When co-expressed only with RAD, DRIF2 is present in the cytoplasm, nucleus and also in the nucleolus (Figure 5m). The co-expression of YFPc-DRIF2 and YFPn-RAD confirmed that DRIF2 and RAD interact in the cytoplasm, nucleus and in the nucleolus. However, DRIF2 is absent from the nucleolus in the presence of both RAD and DIV (Figure 5n). These results suggest that DRIF2 might still interact with DIV inside the nucleus in the presence of RAD. Nevertheless, like in the case of DRIF1, RAD is able to retain some of the DRIF2 protein in the cytoplasm.

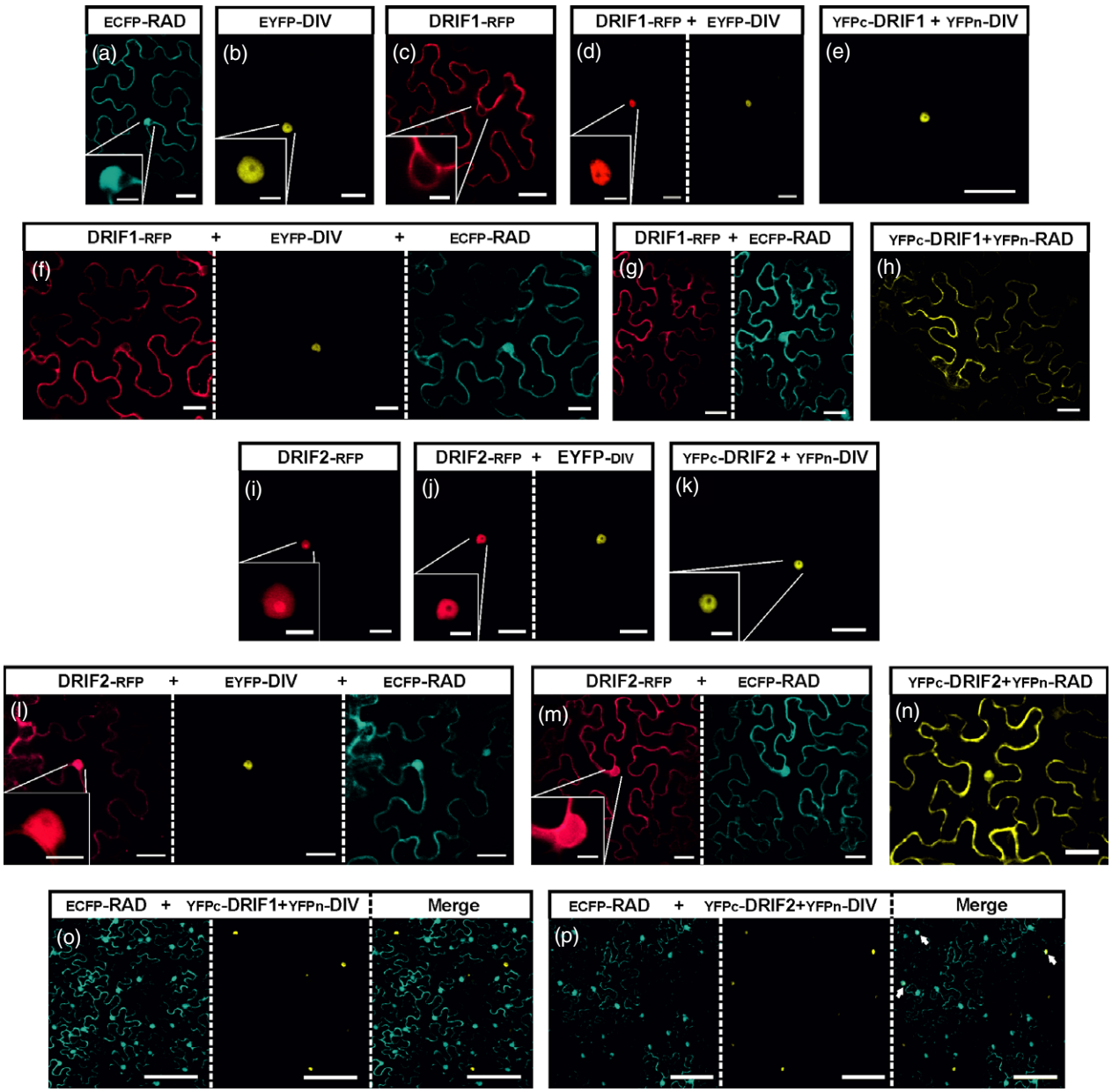

Figure 5. Subcellular competition between RAD and DIV for the DRIF proteins.
Agro-infiltrations of *Nicotiana benthamiana* leaf epidermal cells with different combinations of fluorescent tagged protein constructs.
(a) Expression of ECFP-RAD, localised in the cytoplasm, nucleus and inside the nucleolus (inset). (b) Expression of EYFP-DIV, observed only inside the nucleus but outside the nucleolus (inset). (c) Expression of DRIF1-RFP, showing fluorescence signal in the cytoplasm and around the nucleus (inset). (d) Co-expression of DRIF1-RFP and EYFP-DIV, showing DRIF1 localisation (left, inset) that becomes co-localised with DIV (right) in the nucleus. (e) Bimolecular fluorescence complementation between YFPc-DRIF1 and YFPn-DIV showing interaction in the nucleus. (f) Triple co-expression of DRIF1-RFP, EYFP-DIV and ECFP-RAD, showing the cytoplasmic localisation of DRIF1 (left) identically to what is shown in (c), localisation of DIV (middle) and RAD (right) remains unchanged. (g) Co-expression of DRIF1-RFP and ECFP-RAD, showing DRIF1 localised in the cytoplasm (left) and RAD in the cytoplasm, nucleus and inside the nucleolus (right) identically to the single expressions of DRIF1 and RAD. (h) Bimolecular fluorescence complementation between YFPc-DRIF1 and YFPn-RAD showing interaction in the cytoplasm. (i) Expression of DRIF2-RFP, localised in the nucleus and inside the nucleolus (inset). (j) Co-expression of DRIF2-RFP and EYFP-DIV, DRIF2 signal is visible only in the nucleus (left) but outside the nucleolus (inset) and is co-localised with DIV (right). (k) Bimolecular fluorescence complementation between YFPc-DRIF2 and YFPn-DIV showing interaction in the nucleus but outside the nucleolus (inset). (l) Triple co-expression of DRIF2-RFP, EYFP-DIV and ECFP-RAD, showing a different pattern of DRIF2 cytolocalisation becoming cytoplasmic and nuclear (left) but not nucleolar (inset) which appears to be a combination of both DIV (middle) and RAD (right) cytolocalisations. (m) Co-expression of DRIF2-RFP and ECFP-RAD, showing a shift in DRIF2 localisation that becomes cytoplasmic, nuclear and nucleolar (left, inset) identically to RAD (right). (n) Bimolecular fluorescence complementation between YFPc-DRIF2 and YFPn-RAD showing interaction in the cytoplasm, nucleus and nucleolus. (o) Co-expression of YFPc-DRIF1, YFPn-DIV and ECFP-RAD showing that DRIF1 only interacts with DIV in cells which are not expressing ECFP-RAD. (p) Co-expression of YFPc-DRIF2, YFPn-DIV and ECFP-RAD showing that DRIF2 only interacts with DIV in cells that have are not expressing ECFP-RAD or that have a low level of expression (white arrows). Scale bars represent 30 μm except for the insets that represent 10 μm and (o) and (p) that represent 100 μm.

The dynamic cytolocalisation patterns of the DRIF proteins when co-expressed with RAD and DIV are summarised in Table 1 and indicate how the cytolocalisation of DRIFs changes in the presence of both RAD and DIV to different subcellular compartments. In both cases, RAD protein is able to retain the DRIFs in the cytoplasm. This might contribute to antagonising DIV through disruption or reduction in the assembly of nuclear DRIF–DIV transcriptional complexes. To confirm that in the presence of RAD the interaction between the DRIFs and DIV is disrupted a series of BiFC experiments were performed in which the BiFC pairs YFPc-DRIF1/DRIF2 and YFPn-DIV were co-expressed with ECFP-RAD. The results show that the BiFC interaction between DIV and DRIF1 is only detected in the nuclei of the cells that are not expressing ECFP-RAD (Figure 5o), thus confirming that the presence of RAD completely antagonises the formation of DRIF1–DIV complexes. In the case of DRIF2, most cells that are expressing ECFP-RAD do not show an interaction between DRIF2 and DIV. However, some of the cells that have a lower expression level of ECFP-RAD show interaction between DRIF2 and DIV (Figure 5p) indicating that a high level of expression of RAD is needed to completely disrupt the formation of DRIF2–DIV complexes inside the nucleus. These results show that RAD is able to antagonise the formation of DRIF–DIV complexes by sequestering the DRIFs in the cytoplasm and additionally by disrupting the DRIF–DIV interaction inside the nucleus.

Domains of expression of *DRIF*s and *DIV* overlap

RAD might be antagonising DIV in the dorsal regions of the flower by inhibiting the interaction between the DRIFs and DIV, hence negatively affecting DIV transcriptional activity. Therefore, to correctly regulate its target genes, DIV would need to be spatially and temporally associated with the DRIFs. When co-expressed in the same cell, DRIFs and DIV are located in the same cellular compartments. However, it was still unknown whether the *DRIF* genes were expressed in the same organs as *DIV* in the *Antirrhinum* flower. The expression patterns of *DRIF1* and *DRIF2* were therefore compared with those of *DIV* and *RAD* by RT-PCR.

Table 1 Summary of the cytolocalisation dynamic pattern of the DRIF proteins when co-expressed with RAD and DIV

Expressed proteins	Cytolocalisation of the DRIFs
DRIF1	Cytoplasm
DRIF1 + RAD	Cytoplasm
DRIF1 + DIV	Nucleus
DRIF1 + DIV + RAD	Cytoplasm
DRIF2	Nucleus + nucleolus
DRIF2 + RAD	Cytoplasm + nucleus + nucleolus
DRIF2 + DIV	Nucleus
DRIF2 + DIV + RAD	Cytoplasm + nucleus

The expression of *DRIF1*, *DRIF2*, *RAD* and *DIV* was analysed throughout different floral development stages and organs of *Antirrhinum* (Figure 6), including inflorescence apexes, 0.5 cm width buds, 1 cm width buds and the different petals (dorsal, lateral and ventral) of young flowers. Both *DRIF1* and *DRIF2* were expressed at all flower developmental stages analysed and in all petals of young flowers. *DIV* expression was also detected in every sample analysed in a pattern similar to that of the *DRIF*s. On the other hand, *RAD* expression was specific to the dorsal regions of the flower.

This result supports the idea that the *DRIF*s may be important partners for *DIV* transcriptional activity in defining the ventralised phenotype of the flower, which might be antagonised in the dorsal regions of the flower, where *RAD* is expressed.

DISCUSSION

The establishment of dorsal and ventral domains of gene activities in the *Antirrhinum* flower meristem is key to generating an asymmetrical flower. Dorsal identity is conferred by the presence of *RAD*, that confines DIV activity to the ventral domain of the meristem. The molecular mechanism by which the antagonistic action of RAD over DIV is established was unclear. Here, we demonstrate that RAD is likely to antagonise DIV activity by competing for a DRIF protein and sequestering it in the cytoplasm, which in turn prevents or diminishes the interaction between DRIF and DIV.

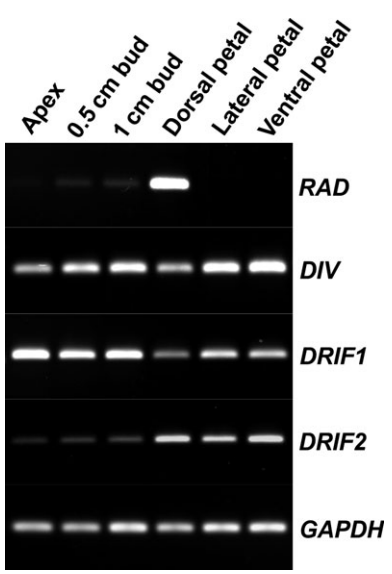

Figure 6. Expression pattern of *DRIF1*, *DRIF2*, *DIV* and *RAD* determined by RT-PCR.
RNA was extracted from wild-type *Antirrhinum* inflorescence apexes, floral buds with 0.5 and 1 cm width and dorsal, lateral and ventral petals from young flowers (1.5 cm width). *RAD* cDNA was amplified with 25 cycles, *DIV* with 28 cycles and the *DRIFs* with 35 cycles. *GAPDH* was used as a control for constitutive expression and was amplified with 25 cycles.

This might be essential in the modulation of DIV transcriptional activity and, consequently, in the establishment of an asymmetric pattern of gene activity in the *Antirrhinum* flower meristem.

Both RAD and DIV are proteins that belong to the MYB protein super family. DIV is a transcription factor that contains two MYB repeats and is capable of binding DNA and activating transcription. A random binding site selection experiment has revealed that DIV is able to bind to an I-box sequence, in agreement with what was shown for the C-terminal MYB domain of another DIV-like protein (Rose *et al.*, 1999). RAD belongs to a small subfamily of proteins containing a single MYB repeat. Interestingly, the RAD MYB domain is very similar to the N-terminal MYB domain of DIV, which is thought to be responsible for protein–protein interactions (Rose *et al.*, 1999). The analysis of the three-dimensional structure of RAD MYB domain revealed that the third α-helix, which binds DNA in other MYB transcription factors, is notably longer in RAD and has no similarity to the DNA-recognition helix of other MYBs (Stevenson *et al.*, 2006). Our results show that, *in vitro*, RAD cannot bind to the DNA-binding site of DIV nor does it prevent DIV from binding. Additional evidence that RAD does not bind DNA comes from a RAD homologue in tomato that is not able to bind DNA on a random population of double-stranded DNA sequences (Machemer *et al.*, 2011). Moreover, we have shown that RAD and DIV do not interact directly either in yeast or and *in planta*. Lack of interaction between DIV and RAD-like proteins seems to be conserved in other species as it was previously shown that six Arabidopsis DIV homologues failed to interact with two Arabidopsis homologues of RAD and the tomato homologue FSM1 in a Y2H assay (Machemer *et al.*, 2011). Therefore, the molecular mechanism for the antagonism that RAD exerts over DIV is most likely processed at the post-transcriptional level involving inhibitory competition for a common interactor rather than competition for a common target DNA or a direct inhibitory interaction between RAD and DIV.

It was shown that, in tomato, a MYB protein (SlFSB1) interacts with a RAD (SlFSM1) and a DIV (SlMYB1) homologue through the MYB-like domain and that SlFSM1 competes for SlFSB1, revealing a new type of antagonistic interaction where two MYB proteins compete for another MYB-like interactor (Machemer *et al.*, 2011). In *Antirrhinum*, we have identified two SlFSB1 homologues, the DRIF1 and DRIF2 proteins, as RAD interactors through a Y2H screening of a cDNA library from *Antirrhinum* flowers. The DRIF proteins are MYB-like proteins that contain a MYB-like N-terminal domain and a domain of unknown function at the C-terminal. These proteins are also able to interact with DIV and, therefore, may be common interactors that are prevented from binding to DIV in the presence of RAD. Curiously, the mechanism used by other single-MYB proteins to antagonise two-repeat MYB proteins seems to have some similarities since they compete for the interaction with a third protein (basic helix-loop-helix co-regulators) in order to specify root hair patterning and trichome development (Wada *et al.*, 1997; Lee and Schiefelbein, 1999; Payne *et al.*, 2000; Bernhardt *et al.*, 2003; Tominaga *et al.*, 2007).

Here, we have shown by gel-shift experiments that DRIF1 and DIV are able to form a protein complex that binds to the DIV DNA-binding site, which suggests that the DRIFs may act as co-regulators of DIV transcriptional activity. Furthermore, the *DRIF* genes are expressed in the same floral regions as *DIV*, supporting the idea that DRIFs and DIV may act as part of a complex in the establishment of the ventral identity of the *Antirrhinum* flower. We further demonstrated that, *in vitro*, the presence of RAD is able to disrupt the formation of a DRIF1–DIV heterodimers bond to DNA, strongly indicating that RAD can prevent the formation of a DRIF–DIV transcriptional complex.

The modulation of the cytolocalisation of regulatory proteins has been shown to be important for several antagonistic molecular mechanisms involved in developmental and defence mechanisms, particularly in plants (Hackbusch *et al.*, 2005; Kaminaka *et al.*, 2006; Froidure *et al.*, 2010; Hong *et al.*, 2011). In the current case, cytolocalisation dynamics is also essential in the antagonistic action that RAD has over DIV by competing for the DRIF proteins. We have demonstrated that subcellular localisation of the DRIFs is regulated by the presence of both RAD and DIV. DRIF1 is a cytoplasmic protein, but when co-expressed with DIV both proteins interact and DRIF1 becomes nuclear. DRIF2, on the other hand, is localised in the nucleus and the nucleolus, but in the presence of DIV it is excluded from the nucleolus. Therefore, DIV is able to shuttle the DRIF proteins from different subcellular localisations to the nucleus. The presence of RAD sequesters the DRIF proteins in the cytoplasm, preventing or reducing the formation of DRIF–DIV heterodimers in the nucleus, which might negatively regulate DIV transcriptional activity.

Although RAD is able to sequester and interact with both DRIF1 and DRIF2 in the cytoplasm, some of the DRIF2 protein interacts with RAD also in the nucleus and nucleolus. However, considering that *in vitro* RAD is able to prevent the DRIF1 protein from binding to DIV when the three proteins are together, and that RAD interacts directly with DRIF2 both in the cytoplasm and inside the nucleus, it can be postulated that the amount of DRIF2 protein that RAD sequesters in the cytoplasm, together with a possible partial disruption of DRIF2–DIV dimers inside the nucleus, might be enough to critically decrease the transcriptional activity of DIV. This might be important in *Antirrhinum* to antagonise DIV in the dorsal domain of the flower meristem generating distinct domains of DIV transcriptional activity. In tomato, the cytolocalisation dynamics of SlFSB1,

a DRIF homologue, resemble the subcellular dynamics of the DRIF2–RAD interaction. SlFSB1 is a nuclear protein that becomes partially retained in the cytoplasm when co-expressed with the RAD homologue, but it is not known whether in tomato the DIV homologue is localised to the nucleus or if it is able to influence the subcellular localisation of SlFSBl. Curiously, a similar type of molecular antagonism involving the nuclear import of regulatory proteins has been shown to occur in *Drosophila melanogaster* to establish the dorsoventral asymmetry of the embryo (Reeves and Stathopoulos, 2009). The dorsoventral pattern is established by a gradient of the activity of the Dorsal protein which defines the ventral identity of the *Drosophila* embryo. In the ventral regions of the embryo, Dorsal is inside the nucleus and is able to activate the genes responsible for the ventral identity. However, in the dorsal regions of the embryo, the Cactus protein sequesters Dorsal in the cytoplasm and prevents it from activating the ventral identity genes, thus promoting the dorsality of the *Drosophila* embryo.

The phylogenetic analysis of the DRIF protein family revealed that this is an ancient protein family that contains several homologues in angiosperms which can be divided into two distinct clades. The fact that the DRIFs from *Antirrhinum* are both placed on the group 1 clade suggests that *Antirrhinum* might have more of these genes yet to be identified. Other *RAD-like* and *DIV-like* genes have been identified in *Antirrhinum* (Galego and Almeida, 2002; Baxter *et al.*, 2007) but have not been functionally characterised. It is possible that together with RAD and DIV homologues, the other DRIF proteins may function in a similar molecular module to perform different roles. It will be interesting to determine whether the interactions between these three types of MYB-like proteins have been recruited in this species for different developmental processes, providing a good example of heterotopic expression of existing functions. In tomato, the DRIF homologue SlFSB1 is thought to mediate the antagonism between RAD and DIV homologues to control differential cell growth during tomato fruit development (Machemer *et al.*, 2011). Although SlFSB1 is not the closest DRIF homologue in tomato, it appears to also be the target of the competition between the RAD and DIV homologues in an antagonistic mechanism similar to the flower of *Antirrhinum*.

In conclusion, we propose a molecular model for the antagonism that RAD exerts over DIV in the dorsal regions of the *Antirrhinum* flower (Figure 7). In the ventral region of the flower, DIV interacts with a DRIF protein, supposedly through the DRIF MYB-like domain, promoting its nuclear shuttling. Inside the nucleus, the DRIF–DIV complex is able to promote the regulation of the genes that specify the ventral identity of the petals. In the dorsal regions of the flower, where RAD is expressed, RAD competes with DIV for a DRIF protein, inhibiting its shuttling to the nucleus and possibly preventing the formation of a DRIF–DIV transcriptional complex. Without the presence of an active DRIF–DIV complex, the ventral identity is not determined, which results in the formation of the dorsal identity and the establishment of an asymmetric pattern of gene activity in the *Antirrhinum* flower.

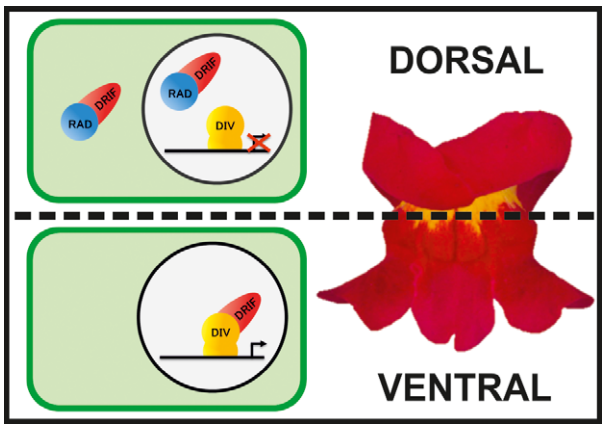

Figure 7. Proposed model for the antagonistic molecular mechanism that RAD exerts over DIV in the dorsal regions of the *Antirrhinum* flower.
In the ventral region of the flower, DIV interacts with a DRIF protein promoting its nuclear localisation. Once in the nucleus, the DRIF–DIV complex is able promote the regulation of genes that specify the ventral identity of the petals.
In the dorsal regions of the flower, RAD competes for a DRIF protein with DIV, promoting DRIF cytoplasmic localisation and decreasing its availability in the nucleus. RAD might also bind to the DRIF protein inside the nucleus thus preventing the formation of the DRIF–DIV transcriptional complex. Without the formation of the DRIF–DIV complex the ventral identity genes are not activated, which results in the formation of the dorsal identity and the establishment of the flower asymmetry in *Antirrhinum*. The similar MYB domains of RAD and DIV are shown with round shapes. Both these domains are assumed to interact with the DRIF MYB-like domain.

EXPERIMENTAL PROCEDURES

Plant materials and growth conditions

Antirrhinum majus wild-type plants (JI7) were grown in the greenhouse as described previously (Carpenter *et al.*, 1987). *Nicotiana benthamiana* plants were grown in a greenhouse with 26/22°C day/night temperature cycles and 16-h light/8-h dark cycles.

Expression and purification of recombinant proteins

For expression of proteins in *E. coli*, the ORF of *DIV* was amplified by PCR from an *Antirrhinum* cDNA library using appropriate primers (Table S1) and cloned into the pRSETC expression vector (Invitrogen, http://www.invitrogen.com/), enabling expression of DIV with an N-terminal 6× histidine tag. The *DRIF1* ORF was amplified from *Antirrhinum* cDNA and cloned into a pGEX6P-1 vector (GE Healthcare, http://www3.gehealthcare.com/), in fusion with an N-terminal GST-tag. A His-tag RAD protein was expressed and purified as described (Stevenson *et al.*, 2006). Recombinant DIV protein was purified from *E. coli* BL21 DE3-pLysS strain (Novagen, http://www.emdmillipore.com/life-science-research/novagen/c_YTKb.s1OFbwAAAEjSGVXhFCX) grown in LB medium supplemented with 2 g L^{-1} lactose for 15 h at 37°C (Machado *et al.*, 2013). DRIF1 recombinant protein was obtained from BL21

DE3-pLysS strain induced with 1 mM isopropyl β-D-thiogalactoside (IPTG) at 37°C for 2 h and purified by affinity chromatography on GST-resin (Clontech, http://www.clontech.com/) according to the manufacturer's instructions.

Random binding-site selection and EMSA

Random binding-site selection with His-DIV protein was performed as described (Costa *et al.*, 2005). A double-stranded DNA probe (36 bp) containing the binding site of DIV was labelled with DIG-11-ddUTP according to the manufacturer's instructions (DIG Gel Shift kit second generation, Roche, http://www.roche.com/). DNA–protein binding reactions were performed in 10 μl reaction mixtures containing 10 ng DNA probe, 10 mM 2-amino-2-(hydroxymethyl)-1,3-propanediol (TRIS)-HCl pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.1% (w/v) BSA, 2 mM DTT, and 4% (v/v) glycerol, 50–500 ng of purified protein, 500 ng poly[d(A-T)]. Reactions were incubated for 15 min at 20°C and loaded on a pre-run 6% native polyacrylamide gel. Electrophoresis was conducted at 8 V cm^{-1} for 45 min with TBE buffer (89 mM TRIS, 89 mM boric acid, 2 mM EDTA, pH 8.0) at 4°C. Transfer and detection was performed according to the manufacturer's instructions.

Yeast two-hybrid analysis

Protein–protein interactions were analysed using a GAL-4-based yeast hybrid system (Matchmaker two-hybrid system; Clontech). *Saccharomyces cerevisiae* strain AH109 (James *et al.*, 1996) was transformed with a *RAD* ORF cloned into pGBT9 (bait vector; Clontech) using the lithium acetate/DNA/polyethylene glycol transformation method (Gietz *et al.*, 1995). The resulting yeast cells were subsequently transformed with prey plasmid DNA derived from an *A. majus* cDNA floral library. Selection of positive interactors was performed according to Causier and Davies (2002).

Subcellular localisation experiments

The ORFs of *DIV, RAD*, *DRIF1* and *DRIF2* were cloned using Gateway technology (Invitrogen) in fusion with different fluorescent proteins. *DIV* was recombined into pH7WGY2 (Karimi *et al.*, 2002), generating an EYFP fusion at the N-terminus, whereas *RAD* was recombined into pH7WGC2, generating an ECFP at the N-terminus. Either *DRIF1* and *DRIF2* entry clones were recombined into both the pH7WGR2 and pH7RWG2 (N-terminal and C-terminal fusion with RFP, respectively).

To perform the subcellular localisation assay, *Agrobacterium tumefaciens* strain GV3101:pMP90 was used to transiently transform leaves of 2- to 4-week-old *N. benthamiana* plants. DIV-EYFP, RAD-ECFP, DRIF1-RFP, DRIF2-RFP, RFP-DRIF1 and RFP-DRIF2 were expressed individually and double or triple co-expressed in all possible combinations. Confocal laser scanning microscopy was performed 1–2 days after infiltration. Three independent repetitions of each infiltration combination were carried out.

Bimolecular fluorescence complementation experiment

For the BiFC experiment the ORFs of *DRIF1*, *DRIF2*, *RAD* and *DIV* were cloned into the vectors pYFC43 and pYFN43 (Belda-Palazón *et al.*, 2012) to generate the YFPc and YFPn N-terminal fusions for all the cloned ORFs. The agro-infiltration of *N. benthamiana* leaves was performed as described above for all the combinations of BiFC pairs.

Analysis of gene expression by RT-PCR

Total RNA was extracted from wild-type *Antirrhinum* using the Qiagen RNeasy RNA extraction kit (Qiagen, http://www.qiagen.com/) and first-strand cDNA was synthesised using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen) on 1.5 μg of total RNA treated with DNaseI. Aliquots of the cDNA diluted 20-fold were used as the template for PCR with cDNA-specific primers (Table S1).

Phylogenetic methods

The DRIF homologous protein sequences were obtained by performing a PSI-BLAST at the NCBI database (http://www.ncbi.nlm.nih.gov/) and by searching the plant gene index database (http://compbio.dfci.harvard.edu/tgi/plant.html) for expressed sequence tag sequences. The protein sequences were aligned with PRANKSTER (Löytynoja and Goldman, 2005). The alignment of the MYB-like and DUF3755 domains were chosen to calculate the distance estimates using the Jones–Taylor–Thornton model of evolution for a neighbour-joining tree with the PHYLIP software package (Felsenstein, 1986). The tree was rooted using the midpoint rooting method. To provide statistical support for each node on the tree, a consensus tree was generated from 1000 bootstrap data sets.

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ACCESSION NUMBERS

Sequence data from this article can be found in the GenBank/EMBL data libraries under the following accession numbers: *DRIF1* protein coding sequence (JX966358); *DRIF2* protein coding sequence (JX966359).

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Data S1. Alignment used to construct the phylogeny of the DRIF proteins presented in Figure 3.

Figure S1. Sequence conservation of DRIF-like proteins.

Figure S2. GST-DRIF1 interacts *in vitro* with ECFP-RAD and EYFP-DIV.

Figure S3. Controls for cytolocalisation and bimolecular fluorescence complementation experiments.

Figure S4. Molecular size confirmation of the fluorescent-tagged proteins.

Table S1. Oligonucleotide sequences.

Methods S1. *In vitro* pull-down assay and Western blotting.

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