Erratum

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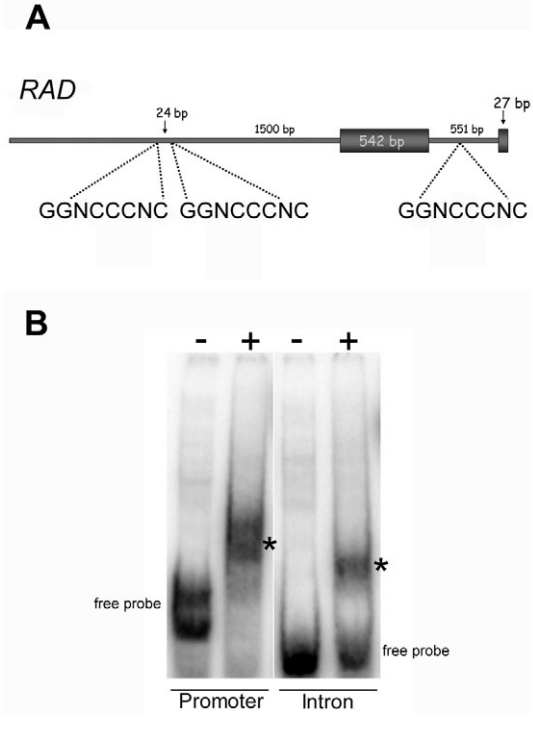
Some errors in this article were not corrected before it was sent to press.

In Fig. 2A, 60 bp should have been 24 bp. The correct figure is printed below.

In addition, in the legend to Fig. 10, the first occurrence of 'CYC' should have been in italics, as it is referring to the gene and not to the protein.

We apologise to the authors and readers for these mistakes.



Evolution of regulatory interactions controlling floral asymmetry

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Summary

A key challenge in evolutionary biology is to understand how new morphologies can arise through changes in gene regulatory networks. For example, floral asymmetry is thought to have evolved many times independently from a radially symmetrical ancestral condition, yet the molecular changes underlying this innovation are unknown. Here, we address this problem by investigating the action of a key regulator of floral asymmetry, *CYCLOIDEA* (*CYC*), in species with asymmetric and symmetric flowers. We show that *CYC* encodes a DNA-binding protein that recognises sites in a downstream target gene *RADIALIS* (*RAD*) in *Antirrhinum*. The interaction between *CYC* and *RAD* can be reconstituted in *Arabidopsis*, which has radially symmetrical flowers. Overexpression of CYC in *Arabidopsis* modifies petal and leaf development, through changes in cell proliferation and expansion at various stages of development. This indicates that developmental target processes are influenced by CYC in *Arabidopsis*, similar to the situation in *Antirrhinum*. However, endogenous RAD-like genes are not activated by CYC in *Arabidopsis*, suggesting that co-option of *RAD* may have occurred specifically in the *Antirrhinum* lineage. Taken together, our results indicate that floral asymmetry may have arisen through evolutionary tinkering with the strengths and pattern of connections at several points in a gene regulatory network.

Key words: Cycloidea, Radialis, Dorsoventral, Gene networks, Atavism, *Arabidopsis*, *Antirrhinum*

Introduction

One approach to studying the evolution of novel traits is to analyse mutants that eliminate the trait. Such mutants have been termed *atavistic* as they give phenotypes resembling the presumed ancestral condition (Cantu and Ruiz, 1985; Hall, 1995). The genes identified by atavistic mutations can provide a convenient starting point for analysing how changes in gene interactions can lead to phenotypic novelty (Babu et al., 2004; Gibson and Honeycutt, 2002; Tautz, 2000). By comparing the network of interactions in species with and without the relevant trait, it should be possible to pinpoint some of the key steps underlying evolutionary change. Here, we adopt this approach for the case of dorsoventral asymmetry in flowers, a trait that is thought to have arisen many times independently during evolution (Donoghue et al., 1998; Endress, 1999; Stebbins, 1974) and for which key regulatory genes giving atavistic mutant phenotypes have been identified.

Several genes controlling floral asymmetry have been identified in *Antirrhinum majus*. *Antirrhinum* flowers are asymmetric along their dorsoventral axis, having distinct dorsal, lateral and ventral organ types. Asymmetry is most evident in the petal and stamen whorls and depends on the action of the duplicate genes *CYCLOIDEA* (*CYC*) and *DICHOTOMA* (*DICH*). *CYC* and *DICH* are both expressed in the dorsal domain of the flower meristem and continue to be expressed at later stages in dorsal floral organs, although expression of *CYC* occurs in a wider region than that of *DICH* (Almeida et al., 1997; Luo et al., 1999; Luo et al., 1996). *cyc dich* double mutants have radially symmetric ventralised flowers, while single *cyc* or *dich* mutants have partially ventralised flowers, consistent with sub-functionalisation of paralogs following the *CYC-DICH* duplication (Gubitz et al., 2003; Hileman and Baum, 2003). Developmental analysis of single and double mutants has shown that *CYC* and *DICH* can enhance or repress organ growth, depending on developmental stage and organ type, with *CYC* having a stronger phenotypic effect than *DICH* (Almeida et al., 1997; Luo et al., 1999; Luo et al., 1996).

CYC and *DICH* encode transcription factors belonging to the TCP family, many members of which influence patterns of plant cell growth and proliferation (Almeida et al., 1997; Crawford et al., 2004; Kosugi and Ohashi, 1997; Kosugi and Ohashi, 2002; Kosugi et al., 1995; Luo et al., 1999; Luo et al., 1996; Nath et al., 2003; Palatnik et al., 2003; Tremousaygue et al., 2003). Members of this family can be grouped into two classes, I and II, based on sequence similarity in the TCP domain and the consensus DNA-binding sequence. A good candidate for a direct target of *CYC* and *DICH* is *RADIALIS* (*RAD*). *RAD* is expressed in the dorsal domain of floral meristems, in a manner that depends on *CYC* and *DICH*. Plants mutant for *RAD* have almost fully ventralised flowers, retaining only slight dorsal identity in their uppermost regions, suggesting that many of the effects of *CYC* and *DICH* are mediated through *RAD*. *RAD* acts antagonistically to *DIVARICATA* (*DIV*), which promotes ventral identity (Almeida et al., 1997; Corley et al., 2005; Galego and Almeida, 2002). *RAD* and *DIV* encode related MYB-like proteins that are thought to compete for common protein or DNA targets.

Unlike *Antirrhinum*, *Arabidopsis* has radially symmetrical flowers. The *TCP1* gene of *Arabidopsis* is the closest homologue to *CYC/DICH* and is expressed asymmetrically in the dorsal domain of young flower meristems and axillary meristems (Cubas et al., 2001). This indicates that the common ancestor of *Antirrhinum* and *Arabidopsis* had a *CYC/TCP1*-related gene that was asymmetrically expressed even though the flowers were presumably radially symmetric. In contrast to *CYC* and *DICH*, *TCP1* is only transiently expressed at very early stages of flower development.

To understand the evolution of asymmetry, we analysed how *CYC* acts in *Antirrhinum* and *Arabidopsis.* We show that CYC binds to DNA, and use random binding site selection to define the consensus binding site. Sequences matching this site are found in the *RAD* promoter and intron, and are bound by CYC, indicating that *RAD* may be a direct target of CYC. CYC is also able to activate the *RAD* gene of *Antirrhinum* in the context of *Arabidopsis* but is unable to activate endogenous RAD-like genes of *Arabidopsis*, which lack sequences identical to the consensus binding site. CYC is nevertheless able to act in *Arabidopsis*, reducing leaf and increasing petal size, through changes in cell proliferation and expansion. By activating CYC protein at specific times, we show that CYC can act at various stages of *Arabidopsis* organ growth. Taken together, our results suggest that ancestral TCP1/CYC-like genes played a role in regulating a network of target genes involved in growth and development. Some of these interactions could have been retained in both *Antirrhinum* and *Arabidopsis,* accounting for the common developmental effects of *CYC* expression in both species. In addition, an interaction between *CYC* and *RAD* was established or preserved specifically in the *Antirrhinum* lineage.

Materials and methods

Plant material and growth conditions

Arabidopsis seeds were sterilised and grown on growth media [GM; 1× Murashige and Skoog salt mixture, 1% (w/v) sucrose, 100 μg/ml inositol, 1 μg/ml thiamine, 0.5 μg/ml pyridoxin, 0.5 μg/ml nicotinic acid, 0.5 mg/ml MES, 0.8% (w/v) agar, pH 5.7] with or without 50 μM kanamycin (Kan). The media was supplemented with 10 μM dexamethasone (DEX; stock solution 25 mM in ethanol) or ethanol 0.04% (v/v) as a control. Plants were grown on a mix of John Innes Potting Compost No 1, vermiculite and grit in the ratio 1.5:1:1 (by volume) at 20°C with 16 hour light/8 hour dark cycles. All *Arabidopsis* transgenic lines (35S::CYC:GR, RAD::RAD) were in the Columbia background, which was also used as the wild-type control.

Production of recombinant CYC protein and electrophretic mobility shift assays (EMSAs)

The pRSET-b vector (Invitrogen) was used for production of recombinant CYC protein in *E. coli* (plasmid pJAM2093). BL21(DE3) SBET pLysE (Schenk et al., 1995; Studier and Moffatt, 1986) *E. coli* cells were transformed with the construct and the recombinant His-CYC fusion protein was expressed under the control of the T7 promoter and purified from the soluble fraction or from inclusion bodies produced in bacteria, as described previously (Kosugi and Ohashi, 1997; Kosugi and Ohashi, 2002). The conditions for the DNA-binding reaction and electrophoresis, and the DNA probes used were as described (Kosugi and Ohashi, 2002). For the EMSA with the *RAD* promoter and intron, the DNA probes were obtained by PCR using the primer combinations 5733/5734 and 5735/5736 with the plasmid pJAM2283, respectively (5733, 5′-ACCGTAGAACATTATAGACAACA-3′; 5734, 5′-CACCAACAA-AACCTTCCACATAG-3′; 5735, 5′-GCTATAACGTCGATGTGT-CTC-3′; 5736, 5′-ATTCTAAAAACCACGAGAGTCC-3′). For the EMSA with the *RAD* promoter and intron, the primer combinations 5733-5734 and 5735-5736 were used, respectively.

Random binding site selection

Random binding site selection for the His-CYC protein was performed using the oligonucleotide BS18N, as described previously (Kosugi and Ohashi, 1997; Kosugi and Ohashi, 2002). The DNA-protein complex was separated by polyacrylamide gel electrophoresis and the DNA recovered from the gel was amplified by PCR. After the fifth round of selection, the DNA amplified by PCR was cloned into pGEMT-easy vector (Promega) for sequencing.

Plasmid construction

The binary plasmid coding for the CYC protein fused to the rat glucocorticoid receptor (GR) (Lloyd et al., 1994) under the control of cauliflower mosaic virus (CaMV) 35S promoter was obtained as follows. The *CYC* open reading frame was amplified by PCR using the primers 5′-CYC (5′-CGGGATCCATGGTTGGGAAG-3′) and 3′-CYC (5′-GAAGATCTTTGATGAACTTGTGCT-3′) from plasmid pJAM2095. The forward primer introduced a Kozac sequence and a *Bam*HI site before the start codon and the reverse primer removed the *CYC* stop codon and created a *Bgl*II restriction site at the C terminus. This PCR fragment was digested with *Bam*HI and *Bgl*II and cloned into the *Bam*HI restriction site of the plasmid GR-pBluescript (pRS020) (Sablowski and Meyerowitz, 1998) resulting in an in-frame translational fusion at the C terminus of CYC with the rat glucocorticoid hormone-binding domain (pJAM2387). The *CYC* cDNA was sequenced to check for PCR errors and to check if the protein was in frame with the *GR* sequence. This fusion was called CYC:GR. The double CaMV 35S promoter and 35S polyadenylation signal from CaMV were isolated as a 1.56 kb *Kpn*I/*Eco*RV fragment from pJIT60 (Guerineau, 1993) and cloned into a *Sac*I filled in/*Kpn*I sites in pGreenII 0029 (plasmid pJAM2388). A 1.7 kb *Xba*I/*Bam*HI fragment containing the CYC:GR fusion was isolated from plasmid pJAM2387 and the ends filled in. This fragment was cloned into the *Sma*I dephosphorylated site of plasmid pJAM2388, adjacent to the double 35S promoter and the orientation was checked by restriction mapping. This construct was called 35S::CYC:GR (plasmid pJAM2389).

Transformation of *Arabidopsis*

Arabidopsis plants were transformed with the 35S::CYC:GR construct by floral dipping (Clough and Bent, 1998). The *Agrobacterium* strain used was C58C1 pGV101 pMP90. Kan-resistant transformants (T1 generation) were selected on GM plates. Approximately, two thirds of T2 plants (segregating 3:1 on Kan) showed a phenotype when also germinated on DEX. T3 progeny of plants that showed a phenotype on DEX-containing media were hemizygous, whereas the progeny of plants that did not show a phenotype were homozygous. 35S::CYC:GR homozygous plants were crossed to other homozygous independent lines and to wild type. Double hemizygous plants never showed a phenotype when grown in DEX-containing media, whereas the hemizygous lines obtained from the cross with wild type showed the phenotype.

Analysis of expression by northern blot and by RT-PCR

For Northern-blot analysis, total RNA was extracted with TRI-Reagent (Sigma), according to the manufacturer's instructions, from 35S::CYC:GR T2 seedlings grown on Kan and DEX, with and without a phenotype. The RNA was blotted onto Hybond N+ (Amersham) according to the manufacturer's instructions and probed with *CYC* or *NPTII* (neomycin phosphotransferase II) probe.

For RT-PCR analysis, RNA was extracted using RNeasy Plant mini kit (Qiagen), from duplicate samples of tissue from 21-day-old 35S::CYC:GR RAD::RAD plants grown on GM media, 6, 18 and 48

hours after transfer to 10 μM DEX plates. cDNA was prepared from 2 μg of total RNA using Thermoscript RT-PCR system (Invitrogen). Aliquots of the cDNA were used as template for PCR with gene-specific primers.

Phenotypic analysis

Scanning electron microscopy (SEM) analysis was carried out on '2-Ton' epoxy (Devcon) replicas of *Arabidopsis* petals and leaves as previously described (Carpenter et al., 1995; Green and Linstead, 1990). These replicas were sputter coated with gold palladium, analysed and photographed with a Philips XL 30 FEG SEM. Petal and leaf areas were calculated using a program written in MatLab called CellFinder. Medians were obtained by analysis of variance. The analyses were carried out using the statistical package GenStat for Windows 7th Edition (VSN International, Oxford, UK).

Results

CYC binds to TCP class I binding-sites

To determine whether CYC would bind to the consensus DNA-binding sequences for TCP proteins (Kosugi and Ohashi, 1997; Kosugi and Ohashi, 2002), the *CYC* open reading frame was fused in-frame with a HIS-Tag epitope and the recombinant protein purified from *E. coli* extracts using a metal affinity resin. The protein was present in both the insoluble and soluble fractions. Electrophoretic mobility shift assays (EMSA) were carried out using a 21 bp double-stranded oligonucleotide probe that contained the overlapping consensus binding sites for both class I and class II TCP proteins (GTGGTCCCAC) (Kosugi and Ohashi, 2002). Incubation of the probe with CYC protein from the soluble fraction showed band retardation (Fig. 1A, lane 1), indicating that CYC protein can bind to these DNA sequences. Mutations in the core of the oligonucleotide abolished binding (Fig. 1A, lanes 2 and 3), indicating that these nucleotides were important for interaction with CYC. According to the sequence of the TCP domain, CYC belongs to the class II subfamily of TCP proteins (Kosugi and Ohashi, 2002) and should therefore bind preferentially to a class II binding site GTGGNCCC. However, when various mutations were introduced in the oligonucleotide to test this, the DNA-binding behaviour of CYC protein was more similar to class I than class II proteins (Fig. 1A, lanes 4 and 5). In particular, mutations that disrupt class II binding did not disrupt CYC binding (Fig. 1A, lane 4). It was only when mutations that affect class I binding were introduced that CYC binding was affected, suggesting that despite sequence similarity to class II proteins, CYC DNA-binding specificity resembles that of class I proteins (Fig. 1A, lane 5). To determine the optimal sequence specificity for the binding of CYC protein, we conducted a random binding site selection analysis using CYC recombinant protein. After five rounds of selection, the program MEME (meme.sdsc.edu) was used to align the sequences of the selected oligonucleotides and the consensus binding site GGNCCCNC was obtained (Fig. 1B), confirming that the CYC binding site is more similar to that of TCP class I than class II proteins.

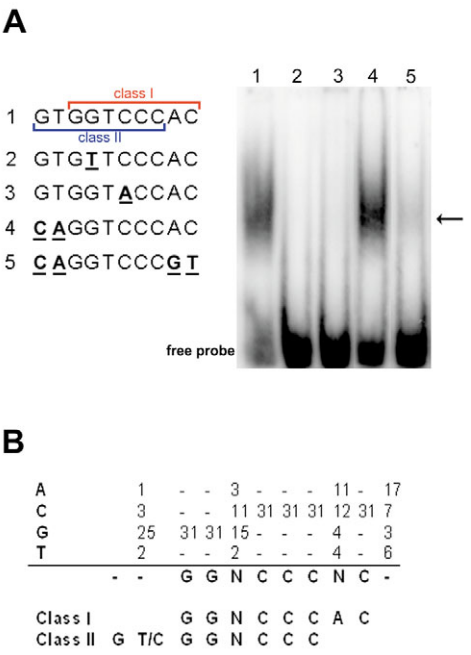

Fig. 1. CYC protein binds to DNA. (A) Specificity of CYC recombinant protein binding to class I and class II TCP consensus binging sites. Oligonucleotide probes contain a shared consensus binding site for class I GGNCCCAC (red) and class II GTGGNCCC (blue) to which CYC protein binds (arrow; lane 1). Bold underlined nucleotides indicate mutations introduced that affect both class I and II binding (lanes 2 and 3) or specifically only class II (lane 4) or class I (lane 5). (B) Consensus DNA-binding sequence for CYC protein obtained by a random binding site selection method. After five rounds of selection, a total of 31 binding sites of CYC were piled up using MEME (meme.sdsc.edu) and a consensus sequence generated. Nucleotides that were not occupied by a specific residue are indicated by N.

In *Antirrhinum*, genetic analysis has suggested that *RAD* could be a potential target gene of CYC (Corley et al., 2005). To investigate whether this interaction could be direct, the *RAD* promoter and intron were screened for CYC-binding sites. Two sites matching the CYC consensus were found 1.5 kb upstream of the start codon of the *RAD* open reading frame, within 24 bp of each other (Fig. 2A). A third matching site was found in the intron. PCR fragments containing promoter and intron putative binding-sites were amplified and used as probes in an EMSA with recombinant CYC protein (Fig. 2B). CYC was able to bind to these sequences, supporting the idea that *RAD* is a direct target of CYC.

CYC affects growth of *Arabidopsis*

To investigate whether CYC expression could affect growth in *Arabidopsis*, the coding region of CYC was fused to that of the rat glucocorticoid receptor and expressed under the control of the 35S promoter (35S::CYC:GR). Sixteen transformants were obtained, of which 10 had a clear developmental phenotype when grown on the glucocorticoid inducer dexamethasone (DEX). Northern-blot analysis on three independent transformants segregating for the construct showed that the severity of the phenotype correlated with the level of *CYC* mRNA accumulation (Fig. 3). Curiously, only plants hemizygous for the construct showed strong expression, indicating that expression was silenced in homozygotes. To check whether this was a consequence of transgene dosage or allelic suppression, 35S::CYC:GR homozygotes were crossed to other independent homozygous lines and to wild type. Double hemizygous plants did not show a phenotype when grown in DEX media, whereas the hemizygous lines obtained

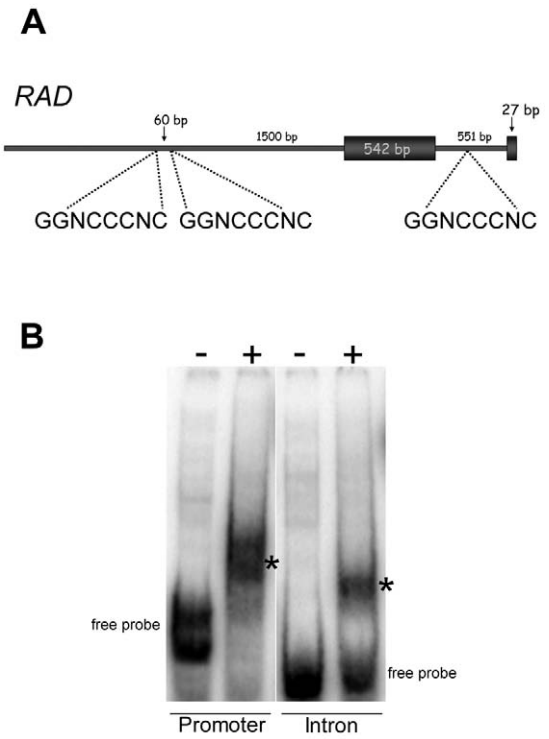

Fig. 2. Interaction of CYC recombinant protein with *RAD* promoter and intron. (A) *RAD* genomic region showing three potential CYC-binding sites in the promoter and intron. (B) PCR fragments of promoter and intron sequences with putative CYC-binding sites were amplified and used as probes in an EMSA with recombinant CYC protein. + or – indicates the presence or absence of CYC protein; asterisks indicate retardation of the mobility of CYC-DNA binding complex.

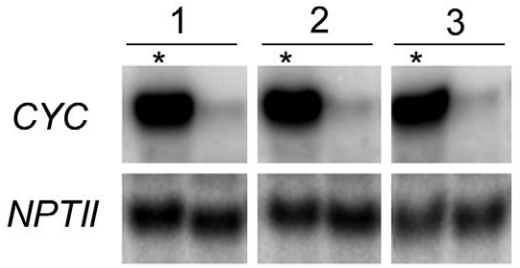

Fig. 3. Northern blot of RNA from 35S::CYC:GR DEX-grown T2 plants derived from three independent transformants, probed with *CYC* (top) or *NPTII* as control (bottom). For each transformant, seedlings were grouped according to whether they had a strong (*) or a wild-type phenotype. Progeny testing showed that in all cases, plants with the strong phenotype were hemizygous, whereas plants with wild-type phenotypes were homozygous for the construct.

from the cross with wild type showed the phenotype. These results indicate that two doses of the transgene result in gene silencing, a phenomenon that has been recorded previously for some transgenes (de Carvalho et al., 1992).

CYC promotes petal expansion in *Arabidopsis*

One aspect of the phenotype of DEX-induced 35S::CYC:GR plants was that the petals were bigger than those of wild type

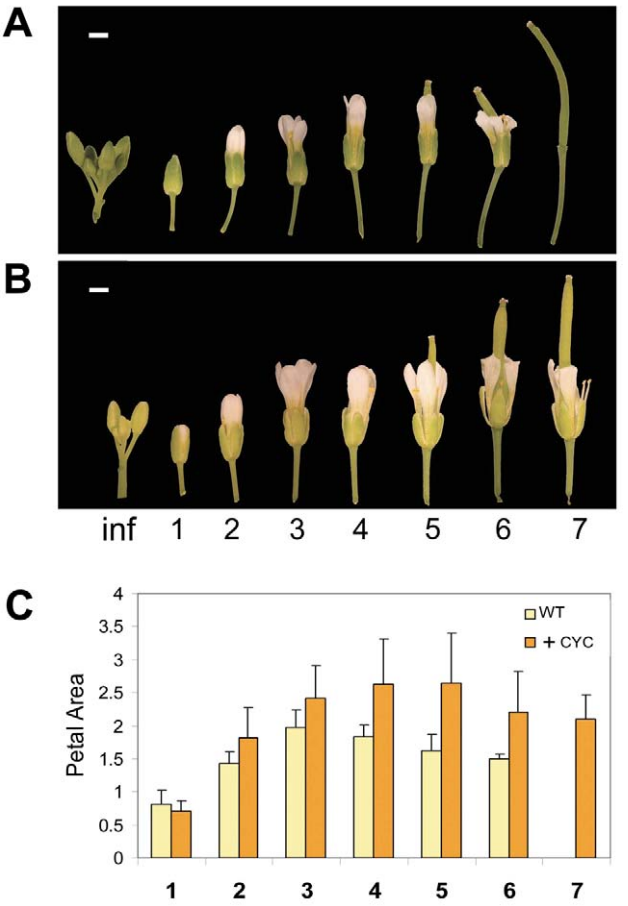

Fig. 4. Effect of CYC in the growth of the petals of *Arabidopsis*. Flowers along the inflorescence of (A) wild-type and (B) 35S::CYC:GR plants grown in DEX. (C) Petal area (mm^2) of consecutive flowers in the inflorescence. Flowers are numbered from the first flower with visible petals (flower 1). The inflorescence remaining after removal of flowers equal or older than stage 1 is labelled 'inf'. Error bars indicate standard deviations. Number of petals measured for each stage was between five and eight. Yellow bars indicate wild-type and orange bars indicate 35S::CYC:GR plants (+CYC) grown with DEX. Scale bars: 1 mm.

(Fig. 4). To determine the stage at which this size change became evident, flowers were staged according to the number of nodes below the first flower with visible petals, termed flower 1 (Butenko et al., 2003; Patterson, 2001). Petals from 35S::CYC:GR flowers became larger than wild-type petals after flower 2 (Fig. 4A-C). Measurements of surface area of mature petals (Fig. 4C) showed that 35S::CYC:GR petals were about 1.5 times larger in area for all four petals. In addition, floral organ senescence was delayed as petals remained attached to the silique for a prolonged period, in contrast to wild-type organs, which abscise at flower 5 or 6. To investigate the correlation between petal area and senescence further, we measured petal areas in two mutants with delayed or deficient abscission: *inflorescence deficient in abscission* (*ida*), in which floral organs remain attached to the plant body after the shedding of mature seeds (Butenko et al., 2003); and *ethylene-response gene* (*etr1-1*), an ethylene response mutant that shows delayed floral organ abscission (Bleecker et al., 1988; Bleecker

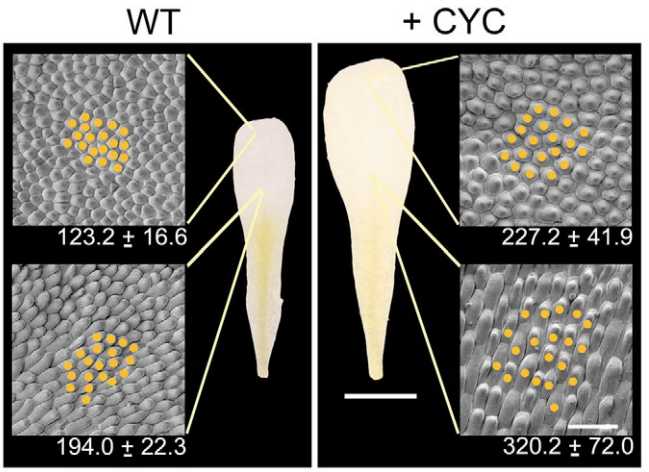

Fig. 5. Effect of CYC on *Arabidopsis* petal cell size. SEM images of the tip and middle areas of wild-type and 35S::CYC:GR petals show that the increase in petal area when CYC is active is due to an increase in cell size. In each SEM image, the same number of cells are highlighted in orange. Numbers indicate cell size ($\mu m^2 \pm$s.e.m.) in the tip and middle regions of the petals, determined using the CellFinder programme. On average between 200 and 500 cells were measured per image and five petals were used to obtain the average cell size in the different petal areas. Left panel shows wild-type and right panel shows 35S::CYC:GR plants (+CYC) grown with DEX. Scale bar: 50 μm on the SEM images; 1 cm on the whole petals.

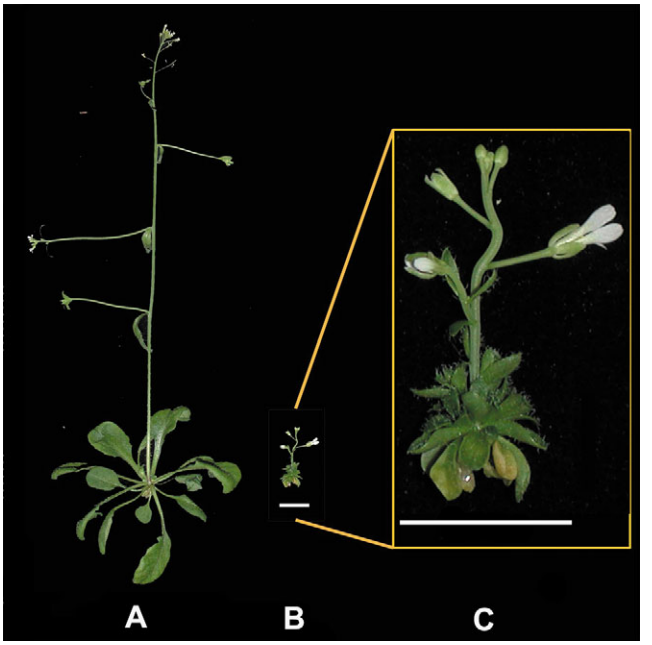

Fig. 6. Effect of overexpressing *CYC* in *Arabidopsis.* 35S::CYC:GR plants have smaller leaves and bigger petals. (A) Wild type; (B) 35S::CYC:GR plants grown on DEX; (C) higher magnification of B (~6×). Scale bars: 1 cm.

and Patterson, 1997; Patterson and Bleecker, 2004). In neither of these mutants was petal size affected to the same degree as 35S::CYC:GR plants, although there may have been a slight increase in size for *etr1-1* (data not shown).

To determine whether the increase in petal area reflected changes in cell proliferation or expansion, the adaxial epidermal layer of mature petals was analysed by SEM (Fig. 5). The average cell area from the tip and middle regions of the petal epidermis was determined. This showed that the cells of 35S::CYC:GR petals were about 1.6 (middle region) or 1.8 (tip region) times bigger than the epidermal cells of wild-type petals. These values are comparable with the overall increase in petal area (1.5 times), indicating that the effect of CYC on petal area could be largely accounted for by increased cell expansion.

To determine the developmental stage at which CYC exerts its effects on petal development in *Arabidopsis*, 35S::CYC:GR plants were transferred to DEX after bolting, when the first two flowers had opened in the main inflorescence. The inflorescence was then allowed to develop further. All flowers showed delayed senescence and abscission, indicating that CYC can have an effect late in development even after the flowers have opened.

CYC represses growth of *Arabidopsis* leaves

35S::CYC:GR plants grown in DEX-containing media, were dwarfed and exhibited small oval-shaped leaves (Figs 6, 7, 8). Mature leaves of 35S::CYC:GR plants were on average eight times smaller in surface area than those of wild type (Fig. 7A,C). To investigate whether the difference in leaf size was due to a difference in cell size or in cell number, the adaxial epidermal layer of leaf 4 was analysed by SEM at different stages of development (Fig. 7B,D). At maturity, cells of 35S::CYC:GR leaves were about four times smaller than those in wild-type plants, suggesting that contrary to what was observed in the petals, CYC has a role in repressing cell expansion in the leaves. However, if reduced cell size was solely responsible for the reduction in overall leaf size, the leaves of 35S::CYC:GR plants should be four times smaller rather than observed value of eight times. This indicates that 35S::CYC:GR leaves also had about half the number of cells of wild type. To determine whether this reflected an early arrest of cell proliferation, epidermal cells of younger leaves were analysed. At day 14, cells of 35S::CYC:GR leaves were larger than those of wild type, suggesting that cell division had arrested early and the cells were starting to differentiate (Fig. 7B,D). Moreover, evidence of recent cell divisions could be seen in the epidermal cells of wild-type leaves (formation of new stomata), whereas in 35S::CYC:GR leaves no such events could be seen. Thus, CYC reduces both cell proliferation (by promoting early arrest of cell division) and cell expansion in leaves.

To determine the developmental stages at which CYC can affect leaf growth, 35S::CYC:GR plants were grown in media with DEX and transferred to media without DEX at day 10 and left to grow to maturity (Fig. 8B). At the time of transfer, the first two rosette leaves were about 2 mm wide and the third leaf was 0.5 mm wide. Analysis of consecutive rosette leaves of mature plants showed that from the third leaf onwards, the leaves increasingly resembled leaves of plants grown continually in the absence of DEX (compare Fig. 8A with 8B).

This showed that removal of CYC could restore leaf growth at early stages of development, before they were about 0.5 mm wide. Moreover, the effect was greater the earlier that CYC was removed.

Conversely, when plants were grown without DEX and moved onto DEX at day 10 (Fig. 8C), growth repression was first detected for leaves 3-4 and became progressively more pronounced in later leaves (compare Fig. 8C with 8D). These results suggest that activation of CYC can influence leaf development at early stages, before leaves are 0.5 mm wide, leading to a reduction in final leaf size.

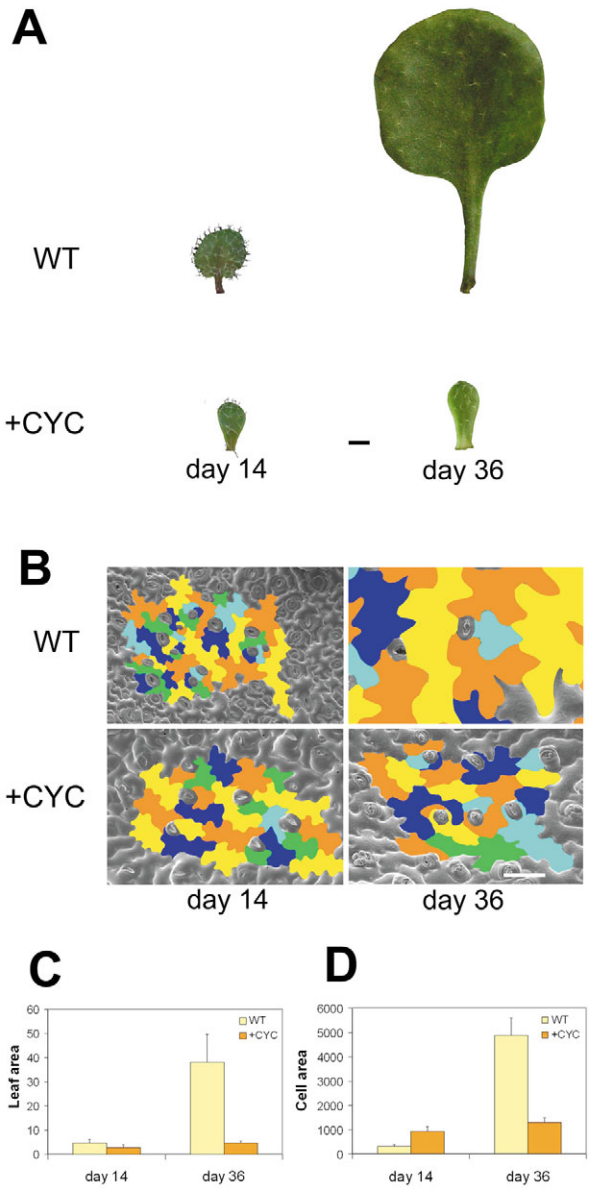

Fig. 7. CYC represses leaf growth in *Arabidopsis*. (A) Leaves from wild-type and 35S::CYC:GR plants grown on DEX (+CYC), at early (day 14; left) and late (day 36; right) developmental stages. In all cases, the fourth rosette leaf was measured. Scale bar: 1 mm. (B) SEM images of adaxial epidermal cells from leaves at the same developmental stage as in A with individual cells highlighted in diverse colours. Scale bar: 50 μm. (C) Measurements of leaf area (mm^2) and (D) epidermal cell area (μm^2) show that CYC reduces leaf surface area by a reducing cell proliferation and cell expansion. Three to five leaves were measured and the area of 19-87 cells in each leaf was obtained. Error bars indicate standard deviations.

CYC activates *Antirrhinum RAD* in *Arabidopsis* but not endogenous RAD-like genes

The *Arabidopsis* genome contains six *RAD-like* genes none of which containing the consensus CYC binding-site within 2 kb upstream or downstream of the coding regions (C.B., M.M.R.C. and E.C.). To investigate whether the effects of CYC in *Arabidopsis* might nevertheless involve activation of these genes, RT-PCR was performed on 35S::CYC:GR plants 6, 24 and 48 hours after being transferred to DEX-containing media. No increase in expression of the RAD-like genes was observed, suggesting that CYC does not affect expression of any of these genes.

To test whether CYC could activate the *Antirrhinum RAD* gene in the context of *Arabidopsis*, 35S::CYC:GR *Arabidopsis* plants were crossed to transgenic plants containing *RAD* under the control of its own promoter (RAD::RAD) (C.B., M.M.R.C. and E.C.). When plants with both transgenes were grown in DEX, they showed the same phenotype as 35S::CYC:GR plants. The double transgenics were also grown without DEX and transferred, at 21 days after germination, to media containing DEX. Fig. 9 shows that *RAD* expression was upregulated 6 hours after the induction of CYC, confirming that CYC can bind to the *RAD* promoter in vivo, and showing that this interaction can be reconstituted in *Arabidopsis*.

Discussion

We have shown that CYC most likely acts as a transcription factor that binds to the *RAD* target gene. The interaction

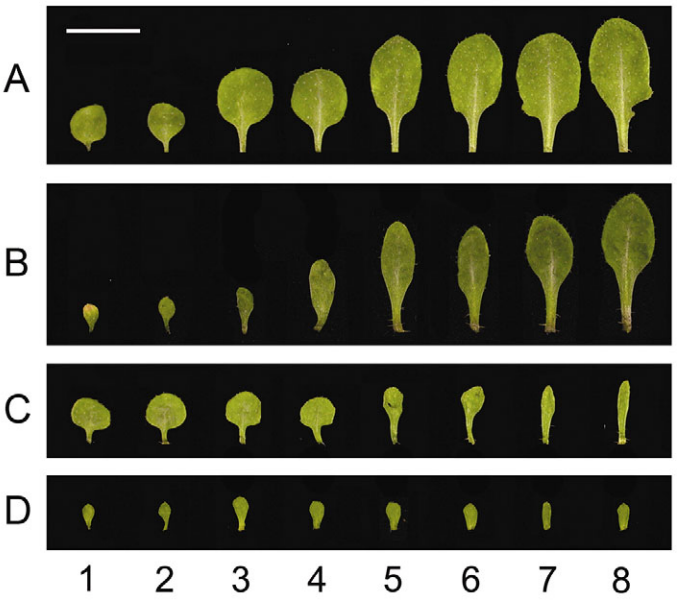

Fig. 8. Effect of CYC activation or inactivation on the growth of *Arabidopsis* leaves. (A,B) 35S::CYC:GR plants were grown in media without DEX (A) or with DEX (B) and left to grow to maturity. (C,D) Alternatively, 10 day after germination, seedlings in which leaf 3 was less than 0.5 mm wide were transferred from media without DEX to media with DEX (C) or from media containing DEX to media without DEX (B). Numbers correspond to consecutive rosette leaves in the mature plants. Scale bar: 1 cm.

between CYC and *RAD* can be reconstituted in *Arabidopsis*, as induction of CYC leads to increased expression of the *Antirrhinum RAD* gene in *Arabidopsis*. Moreover, CYC expression affects growth and development of leaves and petals in *Arabidopsis*, indicating that it is able to influence developmental target genes in this species, as well as in *Antirrhinum*. In the case of leaves, CYC acts from an early stage in development to inhibit cell proliferation and at a later stage to reduce cell expansion, resulting in an overall reduction in leaf size. In petals, CYC acts at a late stage to promote cell expansion, leading to an increase in size of all petals.

In contrast to the situation in *Antirrhinum*, endogenous RAD-like genes are not activated by CYC in *Arabidopsis.* This suggests that although CYC can exert effects in *Arabidopsis*, the network of interactions involving *CYC* has changed since the *Antirrhinum* and *Arabidopsis* lineages diverged. The most recent common ancestor of *Arabidopsis* and *Antirrhinum* most likely had a *TCP1/CYC* gene that was asymmetrically expressed in axillary meristems (Cubas et al., 2001). How might the network of interactions have diverged in the lineages derived from this common ancestor?

In considering the evolutionary changes in a regulatory network, it is convenient to distinguish between two types of target process influenced by a transcription factor. One is the set of potential targets that could be influenced by expression of the transcription factor at any developmental stage and in any part of the plant. This class of targets can be revealed through constitutive expression of the transcription factor. Evolutionary changes in potential targets may occur, for example, through changes in the structure of the transcription factor (i.e. binding site) or changes in the promoters of target genes. The other type of target process is the set of actual targets that are influenced only where and when the transcription factor is normally active. Given a set of potential targets, changes in the expression pattern of the transcription factor can lead to modifications to the defined subset of actual targets.

The ability of CYC to influence both *Antirrhinum* and *Arabidopsis* development suggests that a range of potential developmental targets were present in their common ancestor. A subset of these would have been the ancestral actual targets in the dorsal regions of axillary meristems. Although these actual targets are unknown, they are unlikely to have involved morphological asymmetry of the flower as the ancestral condition is thought to have been radial symmetry.

A key step in the lineage leading to *Antirrhinum* may have been co-option of *RAD* into the network of potential targets (Fig. 10, step a). This could have arisen though mutations in the *RAD* promoter or intron, bringing *RAD* under the control of *CYC*. This would account for why over-expression of CYC in *Arabidopsis* does not lead to activation of RAD-like genes (as they lack appropriate recognition sites) even though it can activate the *Antirrhinum RAD* gene. It is also consistent with the more restricted role of *RAD* in floral symmetry compared with *CYC* and *DICH.*

In addition to co-option of *RAD*, other alterations in the regulatory interactions may also have occurred in the *Antirrhinum* lineage. For example, *CYC* has developmental effects on stamen arrest as well as a slight effect on petal lobe asymmetry independent of *RAD*. These effects may have arisen through divergence in some of the target processes influenced by *CYC* (Fig. 10, step b). It is also possible that the target sequence recognised by CYC diverged to some extent (the CYC consensus binding site is not typical of type II TCP proteins).

Another evolutionary change may have involved persistent expression of *CYC* in the dorsal domain of *Antirrhinum* floral meristems [in contrast to transient expression of *TCP1* in *Arabidopsis* (Cubas et al., 2001)], leading to a change in expression or range of actual targets. Given the phenotypic effects of CYC on petal size in *Arabidopsis*, it is possible that such persistence could create an asymmetric *Arabidopsis* flower with larger dorsal petals. Persistence in the *Antirrhinum* lineage may have arisen through evolution of an auto-regulatory loop (Fig. 10, step c), as *CYC* contains promoter sequences matching the consensus CYC-binding site (the

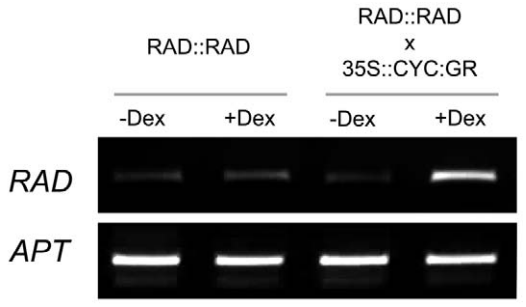

Fig. 9. CYC upregulates *RAD* expression in *Arabidopsis*. RT-PCR analysis of *RAD* transcript in 35S::CYC:GR RAD::RAD plants transferred to DEX. RNA was extracted after 6 hours after transfer. *APT* (adenosine phosphotransferase) was used as a constitutive control.

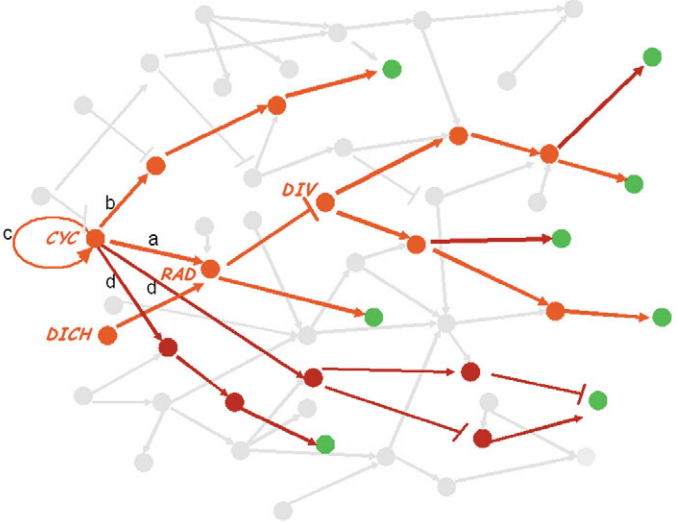

Fig. 10. Schematic representation of evolution of gene interactions in the *Antirrhinum* lineage controlling flower dorsoventral asymmetry. Potential direct and indirect targets of CYC are highlighted in orange and red, with the subset of actual targets indicated in orange. Downstream targets regulating growth are in green and the rest of the regulatory network in which *CYC* interactions are embedded is shown in grey. Steps where divergence involving *CYC* interactions are lettered a-d (see text for explanation).

TCP1 promoter does not contain such sites). However, persistent expression could also have arisen through interaction with other factors that are not directly dependent on *CYC*. In addition, we cannot rule out the possibility that the ancestral *CYC/TCP1* gene was persistently expressed in dorsal floral meristems and that expression became transient in the lineage leading to *Arabidopsis.*

Compared with *CYC*, little is known about the role of *TCP1* and its downstream targets in *Arabidopsis.* Nevertheless, it seems that some of the potential target processes influenced by *CYC* may be conserved in *Arabidopsis*. Expression of CYC in leaf primordia influences both cell division and expansion. Moreover, this effect is cumulative with increased duration of CYC expression. This is comparable with the effects of *CYC* on organ development in *Antirrhinum*, consistent with some overlap between potential target processes influenced by CYC in the two species but also some divergence (Fig. 10, step d).

Expression of CYC also influences petal development in *Arabidopsis*. All petals are equally affected, reflecting ectopic expression of CYC all around the developing flower. The effect of CYC largely involves cell expansion late in development, in contrast to *CYC* in *Antirrhinum*, which also has early developmental effects. Moreover, CYC expression does not affect stamen development in *Arabidopsis,* unlike the situation in *Antirrhinum.* Thus, the potential developmental targets in floral organs have probably diverged, consistent with the inability of CYC to switch on RAD-like genes in *Arabidopsis*.

Taken together, our results indicate that interactions involving *CYC* have changed in many ways since the separation of the *Antirrhinum* and *Arabidopsis* lineages. These changes may themselves represent a subset of the changes that have occurred in the whole regulatory network in which *CYC* is embedded. Nevertheless, some elements of the network have been preserved, such as the initial asymmetric expression pattern and the modification of developmental target gene activity. Thus, floral asymmetry has most likely arisen through a process of tinkering (Jacob, 1977; Jacob, 2001) with the strengths and pattern of connections in a regulatory network, in which some common elements may still be discernable.

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