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Instruments of Functional Genomics for the Improvement of Flower Characteristics in Ornamentals

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María Manchado Rojo

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Instruments of Functional Genomics for the Improvement of Flower Characteristics in Ornamentals

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Directores de la Tesis Doctoral
Dra. Julia Weiss
Dr. Marcos Egea Gutiérrez-Cortines
Departamento de Ciencia y Tecnología Agraria
Área de Genética
Universidad Politécnica de Cartagena

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INFORMA:

Que la referida Tesis Doctoral, ha sido realizada por Dª. María Manchado Rojo, dentro del programa de doctorado Técnicas Avanzadas en Investigación y Desarrollo Agrario y Alimentario, dando mi conformidad para que sea presentada ante la Comisión de Doctorado para ser autorizado su depósito.

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Fdo.: Julia Weiss

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Fecha: 2015.06.15 18:57:01 +02'00'

Fdo: Dr. Francisco Artés Hernández

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A mi familia,

A los que están
A los que vendrán
Y a los que ya se han ido

Resumen

El presente trabajo pretende explorar las técnicas de genética inversa para mejora de la planta ornamental. Se ha desarrollado un protocolo de transformación de *Antirrhinum majus* siéndonos de gran ayuda el desarrollo de una técnica para genotipar cepas de laboratorio de *Agrobacterium tumefaciens* y de *Escherichia coli* basado en los datos de fusionado de ADN de un fragmento de 23S ADN ribosomal, dado que la contaminación cruzada de stocks de *Agrobacterium tumefaciens* con *Escherichia coli* son difíciles de identificar por técnicas microbiológicas, dando lugar a resultados de falsos negativos en los experimentos de transformación.

Para la mejora de planta ornamental, nos hemos basado en el estudio de genes que afectan sobre todo al desarrollo floral en dos especies que son tradicionalmente utilizadas como plantas modelo, pero que además poseen un elevado interés económico, éstas son *Antirrhinum majus* y *Petunia* x *hibrida.*

Se analizaron los niveles de *DEFICIENS* y *GLOBOSA* en las etapas finales del desarrollo de pétalos en los mutantes *compacta*, *deficiens*^{nicotianoides} y *globosa-1* de *Antirrhinum majus*. Nuestros resultados muestran que el nivel umbral de *DEF* o *GLO* para obtener tejido de pétalo es aproximadamente un 11% del silvestre. Demostrándose que en las etapas finales del desarrollo de pétalo, la topología de la red transcripcional de la function B no está basada en la autoregulación positiva y tiene componentes adicionales de mantenimiento de la transcripción.

Comprobamos el uso de *AINTEGUMENTA* como herramienta para modificar el tamaño floral en dos plantas diferentes, *Petunia x hybrida* y *Antirrhinum majus*. La disminución de la expresión de *PhANT* muestra un efecto en el tamaño de las células, mientras que la sobreexpresión de *AtANT* en limbo y tubo de *Petunia* y en *Antirrhinum* causa un significante incremento en la expansion celular que podría explicar las diferencias en el tamaño de los órganos florales. El efecto diferencial de *AtANT* en el limbo y tubo de *Petunia* y *Antirrhinum* corresponde a diferencias fenotípicas observadas en la variación natural en el género correspondiente indicando una relación entre el espacio fenotípico de un género y el efecto de los niveles de *ANT* modificados, validando *ANT* como un gen para modificar el tamaño floral.

Summary

This present work aims to explore the technique of reverse genetics for the improvement of ornamental plants. For this purpose we have developed an *Antirrhinum majus* transformation protocol. The development of a genotyping assay for *Agrobacterium tumefaciens* and *Escherichia coli* lab strains based on DNA melting profiles of a 23S rDNA fragment has been of great help in the process of elaborating the transformation assay, since cross contamination of *Agrobacterium tumefaciens* stocks with *Escherichia coli* are difficult to identify by microbiological techniques, leading to false negative results in transformation experiments.

For the improvement of ornamental plants, we concentrated on the study of genes that affect mostly the floral development in two species which are traditionally used as a model plants, but at the same time are of high economic interest, *Antirrhinum majus*, and *Petunia* x *hybrida*.

We investigated the levels of *DEFICIENS* and *GLOBOSA* at late stages of petal development in the *Antirrhinum majus* mutants *compacta*, *deficiens*^{nicotianoides} and *globosa-1.* We show that the threshold levels of *DEF* or *GLO* to obtain petal tissue are roughly 11% of wild-type. Our results demonstrate that at late stages of petal development, the B function transcriptional network topology is not based on positive autoregulation and has additional components of transcriptional maintenance.

We also tested the use of *AINTEGUMENTA* as a tool to modify floral size in our two model plants, *Petunia x hybrida* and *Antirrhinum majus*. Downregulation of *PhANT* showed an effect on cell size while overexpression of *AtANT* in *Petunia* and *Antirrhinum* caused significant increases in cell expansion that could explain the differences in floral organ size. The differential effect of *AtANT* on limb and tube in *Petunia* and *Antirrhinum* correspond to phenotypic differences observed in natural variation in the corresponding genus indicating a relation between the phenotypic space of a genus and the effect of modified *ANT* levels, validating *ANT* as a gene to modify floral size.

Introduction

Introduction

1. Gene Functions and the Advent of Genomics

The development of the so called 'omics technologies' has had a clear impact on the attention given to the research related to biological processes in all areas. The beginning of such technologies is a result of the automation of genomic DNA sequencing.

Genomics is the science and techniques dedicated to the integrated study of the origin, evolution, operation and content of genomes. The analysis of genomes has undergone a boom in recent years, mainly thanks to advanced DNA sequencing technologies and advances in bioinformatics. These advances have resulted in the realization of huge projects to sequence entire genomes of various model organisms and several public servers, such as the NCBI (National Center for Biotechnology Information) that allow free access to the sequences of many organisms. The strong development of genomics has contributed to the progress in different fields of science, such as agriculture, thanks to the discovery of sequences of genes involved in traits of agronomic importance and through comparison of genomic sequences from different organisms.

An innovation introduced in the early nineties was the systematic sequencing of expressed sequence tags or ESTs (Adams *et al.*, 1991).

The results of sequencing and annotation of genomes are deposited in databases. With the idea of unifying all this information in a meaningful way for researchers, the Gene Ontology (GO) project was born in 1998 (Ashburner *et al.*, 2000). Initially this project involved the association of three model organism databases: the *Drosophila* genome database (FlyBase) (Anon, 1999), Mouse Genome Informatics database (MGI) (Blake *et al.*, 2000) and the *Saccharomyces* Genome Database (SGD)(Ball *et al.*, 2000). But nowadays the GO Consortium has grown to include many databases, such as several major genomes of animals, microorganisms and plants (Harris *et al.*, 2004).

The Gene Ontology Consortium (Consortium, 2001) has created three extensive ontologies to describe molecular functions, biological processes, and cellular components, and providing a community database resource that supports the use of these ontologies:

- Biological process ontology: describes the sets of molecular events with a defined beginning and end, pertinent to the function of integrated living units such as cells, tissues, organs and organism. The biological process ontology may be inferred based on phylogenetic tools due to

the fact that any protein has a specific biological function and a biological pathway requires the systematization of many proteins. Although it requires proof of concept by gain and/or loss of function of the genes involved.

- Molecular function ontology: refers to the molecular activity of a gene product. This can be inferred from BLAST (Altschul *et al.*, 1990), a search algorithm used for comparing biological sequences and finding the similarity between them.
- Cellular component ontology: is defined as the part of a cell or its extracellular environment where a gene product is active. This ontology might be also inferred from BLAST.

2. Reverse Genetics

Traditionally, forward genetics tries to find out the sequence of a gene with known biological function starting from a mutant or an allele with a phenotype. In the last years, the widespread of molecular biology techniques has facilitated the massive sequencing of genes. This fact coupled with a significant cost reduction has enabled the completion of several genome projects. Therefore, nowadays a large number of sequences with an unknown function exist. A recent annotation of the *Arabidopsis* genome suggests that of the 27139 identified functional genes, 7592 (28%) are predicted as protein of either hypothetical or unknown function (Brown *et al.*, 2005).

By using reverse genetics it is possible to identify the biological function of these genes. The first step is generating individuals in which the sequence of the gene of interest has been altered, or changes in its expression levels lead to increased or decreased function. There are several techniques of reverse genetics that have been useful and described as follows.

2.1. RNA Interference (RNAi)

RNA interference is a loss-of-function technique, which is based on the biological process through specific mRNA degradation causing post-transcriptional gene silencing. Small RNA molecules are used as templates by a protein complex enabling it to recognize sequences of RNA, which are targeted for degradation. It is thought that RNA interference evolved as mechanism of defence against viruses and transposable elements (Waterhouse *et al.*, 2001). This technology is based on a control system which modulates gene expression composition (Ketting *et al.*, 1999; Tabara *et al.*, 1999) or gene expression (Palatnik *et al.*, 2003) by small RNA or micro RNAs. There have been many studies based on this technique and it is worth highlighting the researches with *Caenorhabditis elegans* (Fire *et al.*, 1998) and *Petunia hybrida* (Napoli *et al.*, 1990), as they were the first works which led to discover this biological pathway.

2.2. Gain-of-Function

The gain-of-function method consists in introducing extra copies of a gene of interest in an individual. Experiments performed to obtain increased levels of gene expression are done in many cases with the constitutive 35S promoter from Cauliflower Mosaic Virus (CaMV) (Benfey and Chua, 1990). This results in a mixture of overexpression and ectopic expression of the gene product. Its wide use is due to the fact that it derives from a potent virus with a wide host range both in angiosperms and gymnosperms, and a stable expression in many organs and at different developmental stages (Holtorf *et al.*, 1995). The induced overexpression of a gene increases its activity and confers a new or enhanced activity of the corresponding protein. This kind of manipulation usually results in a dominant effect. Numerous studies have applied this technique in order to identify gene functions, including *Drosophila* (Rorth *et al.*, 1998) and many plant species (Kondou *et al.*, 2010).

2.3. TILLING (Targeting Induced Local Lesions in Genomes)

This technique is based on the generation of a directed chemical mutagenesis of a population of individuals (McCallum *et al.*, 2000) by a chemical mutagen such as ethylmethanesulfonate or acridine orange. After that, the point mutations are recognized via DNA-screening techniques, which involve the formation of DNA heteroduplexes, cleavage by single stranded nucleases and size separation (Till *et al.*, 2003). This reverse genetics method has been widely used in several species including zebrafish (Draper *et al.*, 2004) and wheat (Slade *et al.*, 2005).

2.4. T-DNA Tagging

T-DNA is a segment of the Ti plasmid from *Agrobacterium* that is transferred into the host plant and causes gene mutations. T-DNA tagging technique is based on this natural process and consists in replacing the tumor-promoting and opine-synthesis genes from the T-DNA by a selectable marker such us an antibiotic resistance gene or a fluorescent marker (*gfp* or *gus* gene). This method has been used to identify the biological function of genes in different organism such as *Arabidopsis thaliana* (Marks and Feldmann, 1989; Koncz *et al.*, 1990), rice (An *et al.*, 2003) or tobacco (Foster *et al.*, 1999).

2.5. CRISPR/CAS (Clustered Regularly Interspaced Short Palindromic Repeats)

This technique is based on the defence mechanism in prokaryotes (Horvath and Barrangou, 2010), similar to RNAi in eukaryotes (Marraffini and Sontheimer, 2010). CRISPRs are short palindromic repeats in the prokaryotic genome, followed by short fragments of spacer DNA, which are integrated after a virus infection. Together with the CAS protein, they constitute the prokaryotic immune

system allowing prokaryotes to recognize exogenous genetic material and cut it, thus preventing infection. The CRISR/CAS technology is being implemented for targeted genome engineering in higher plants such as crop plants (Belhaj *et al.*, 2015). It is based on coexpression of Cas9 nuclease and an engineered single guide RNA that specifies a targeted nucleic acid sequence. Recent studies have shown its usefulness in humans and mice (Cong *et al.*, 2013) and zebrafish (Hwang *et al.*, 2013).

3. Model Plants and Plants of Industrial Interest

Model organisms are those that possess properties that facilitate growth and reproduction in laboratory conditions. They are usually robust organisms, with a short reproductive cycle and relatively small genomes. These model organisms are studied in biology to understand particular biological processes, which can be then extrapolated to other individuals. In molecular biology and plant genetics various model organisms are used, including *Arabidopsis thaliana*, *Lotus japonicas* or *Picea abies*.

Among the model plants there are some that also have a high agronomic and/or industrial interest. From the point of view of ornamental crops we could highlight species such as *Antirrhinum majus* and *Petunia hybrida.*

Arabidopsis thaliana as a single model plant is unable to represent the wide range of existing angiosperms. *Antirrhinum majus* is considered an alternative model angiosperm plant and the variety of species from *Antirrhinum* makes this plant of great interest for studying variation, inheritance and development (Schwarz-Sommer *et al.*, 2003). *Antirrhinum majus* belongs to the Scrophulariaceae family, native to the Mediterranean region. It was first described as a model plant in the XXth century by Erwin Baur. A total of 500 mutants were described by Hans Stubbe (Stubbe, 1966) and this mutant collection is currently held in Gatersleben (Germany). *Antirrhinum majus* is a popular ornamental plant and one of the classic models for research in floral development (Carpenter and Coen, 1990). *Antirrhinum* flowers are zygomorphic, with only one plane of symmetry, and are composed of five sepals, five petals, four stamens and two fused carpels.

The ornamental garden *Petunia* (*Petunia x hybrida* Hort. ex Vilm., also presented as *Petunia hybrida*) is a hybrid between *Petunia axilaris* and *Petunia integrifolia*, that has also widely been used as a model system due to the availability of molecular genetics tools including a transformation protocol (Gerats and Vandenbussche, 2005). *Petunia* flowers present five sepals, five fused petals, five stamens and two carpels with zygomorphic symmetry (Rijpkema *et al.*, 2006)

4. Floral Development

Flower development requires the transformation of a vegetative meristem into a floral meristem in sexually mature plant. Organ identities are than established within the floral meristem that give rise to the several organs that compose the flower. Flower organs are organized in four concentric rings called whorls. From the outer to the inner these are: sepals, petals, stamens and carpel. The identity of the organs that constitute the flower is determined by three conserved genetic functions, which act in combination and are described by the ABC model (Figure 1). It suggests that A function genes control the identity of sepals, while a coexpression of A and B function genes controls the identity of petals, stamens result from the coexpression of B and C function genes, and carpels by the expression of C function genes (Haughn and Somerville, 1988). The existence of additional D and E function genes was proposed later (Figure 1). The D function specifies the identity of ovules (Colombo *et al.*, 1995) whereas E function would be necessary for the proper development of the four whorls (Ditta *et al.*, 2004). Floral organ development begins with the activation of the so-called organ identity genes. In *Antirrhinum majus* these genes correspond to the MADS-box genes *DEFICIENS* and *GLOBOSA,* that determine the identity of the petals and stamens, and *PLENA,* that controls the stamen and carpel identity (Egea Gutierrez-Cortines and Davies, 2000). *Petunia* is a special case because there has been a complete gene duplication of B function genes involved in petal and stamen identity (Bombarely *et al.*, n.d.). The four B function genes in *Petunia* are: *Petunia hybrida TOMATO MADS-BOX GENE6 (PhTM6),* and *Petunia hybrida DEFICIENS* (*PhDEF)*, paralogs of *APETALA3 (AP3)* and *DEFICIENS*, and *Petunia hybrida GLOBOSA1 (PhGLO1)* and *Petunia hybrida GLOBOSA2 (PhGLO2),* paralogs of *PISTILLATA (PI)* and *GLOBOSA (GLO)* (Rijpkema *et al.*, 2007), being *PhGLO* loss of function alleles those that as single mutants have stronger phenotypes in petal development (Vandenbussche *et al.*, 2004). Petal morphogenesis occurs in several stages with an initial stage of cell division followed by a further stage in which growth occurs by cell expansion. That growth mode is common in *Arabidopsis*, *Antirrhinum*, *Gerbera* and *Petunia* indicating that it is a general growth characteristic of that organ (Egea-Cortines and Weiss, 2013).

A serial of processes are triggered during later stages of floral development that lead to the formation of mature flowers which are able to be pollinated and thereby ensure the continuity of the species. These processes include petal expansion, stamen filament elongation, anther dehiscence and gynoecium maturation. *Arabidopsis* studies revealed that jasmonate plays an important role during late stage in flower development (Brioudes *et al.*, 2009). Mutations in *DEFECTIVE IN ANTHER DEHISCENCE 1(DAD1)* or *DELAYED DEHISCENCE 1 (DDE1)*, two genes involved in the first steps of the synthesis of jasmonic acid, cause defects in anther dehiscence, pollen maturation and floral aperture

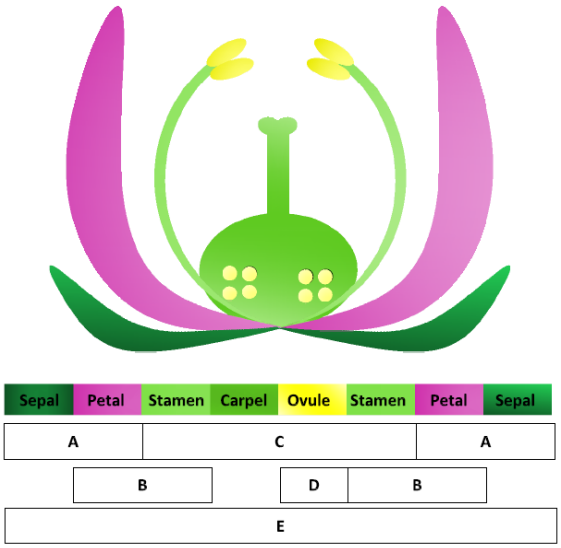

Figure 1: ABCDE model of flower development

in *Arabidopsis* (Sanders, 2000; Ishiguro, 2001). This phytohormone is induced by the transcription factors *AUXIN RESPONSE FACTOR 6* and *8* (*ARF6* and *ARF8*) which trigger the expression of *MYB21* and *MYB24* provoking petal and stamen maturation (Reeves *et al.*, 2012). The *TRANSPORT INHIBITOR RESPONSE 1/AUXIN SIGNALING F-BOX* (*TIR1/AFB)* family of F-box protein is an auxin receptors and studies with *tir1 afb2 afb3* triple and *tir1 afb1 afb2 afb3* quadruple mutants show that auxins are involved in anther dehiscence and pollen maturation (Cecchetti *et al.*, 2008).

Once anthesis is achieved, floral organs start emitting scent. The emission of floral scent occurs from different floral organs including petals, stamens and pistils (Pichersky *et al.*, 1994). It is believed that emission of floral scent is controlled by the type of organ, but direct proof is lacking. Scent emission makes a sudden debut as the profile specific of a species appears as such once flowers open (Weiss *et. al* personal communication). Amongst the volatiles produced by *Antirrhinum* are methylbenzoate, ocimene and myrcene (Wright *et al.*, 2005).

5. Petal Development

The B function genes described in *Arabidopsis thaliana* are *APETALA 3* (*AP3)* (Jack *et al.*, 1992) and *PISTILLATA (PI)*, whereas in *Antirrhinum majus* there are the orthologs *DEFICIENS (DEF)* (Sommer *et al.*, 1990) and *GLOBOSA (GLO)* (Trobner *et al.*, 1992), belonging to the MADS-box gene family of transcription factors.

DEFICIENS is a homeotic gene involved in the control of floral development, concretely in petal and stamen development; hence mutations of this gene lead to sepaloid petals and carpelloid stamens. Three morphoalleles are known in *Antirrhinum* which differ morphologically in the extend of

modification of the different organs (Sommer *et al.*, 1991). *deficiens*^{chlorantha} (*def*^{chl}) has flowers of reduced size, petals with sepaloid features and reduced male fertility due to feminized stamens. Flowers of the mutant d*eficiens*^{nicotianoides} (*def*^{nic}) have greenish sepal-like petals, and badly developed stamens, and *deficiens*^{globifera} (*def*^{gli}) reveals the strongest phenotype (Baur, 1924; Hertwig, 1926). The *def*^{gli} allele has two whorls of sepals and one whorl of carpels, being the fourth whorl suppressed.

GLOBOSA is, as well as *DEFICIENS,* a homeotic gene and since their mutants show a similar phenotype it is thought that both genes have an equivalent function in floral morphogenesis. Similar to *def*^{gli}, the mutant *glo-1* is a null allele that presents petals hardly distinguishable from the first whorl of organs (Tröbner *et al.*, 1992).

MIXTA is a *Myb*-related transcription factor involved in the control of conical epidermal cell shape of *Antirrhinum majus* (Noda *et al.*, 1994). This gene is also expressed in petals, affecting the formation of trichomes (Martin *et al.*, 2002).

The gene *AINTEGUMENTA* (*ANT*) is an *APETALA2* transcription factor, originally identified in a screen for female sterile plants, and is required for ovule initiation in *Arabidopsis* (Klucher *et al.*, 1996; Elliott *et al.*, 1996). Later works have shown that the overexpression of *ANT* in *Arabidopsis* causes increases in organ size while its loss of function results in decreased size because this gene regulates both cell division and cell expansion during the organogenesis (Krizek, 1999; Mizukami and Fischer, 2000). *ARGOS* is an auxin-induced gene that has also been related with organ size control. It acts upstream of *ANT* as an increase in organ size induced by overexpression of *ARGOS* is revoked by the loss of function of *ANT* (Hu *et al.*, 2003). Therefore, *ANT* is a gene downstream of auxin signalling.

6. Control of Lateral Organ Size

6.1. Size

There are many evidences showing that lateral floral organs grow as a result of an initial increase in cell number, followed by cell expansion. This is true in *Arabidopsis* leaves or *Arabidopsis*, *Petunia* and *Gerbera* petals (Laitinen *et al.*, 2005; Reale *et al.*, 2002; Kazama *et al.*, 2010; Anastasiou *et al.*, 2007). The organs appear to have a specific time, during which they have to reach a certain number of cells before proceeding to cellular expansion. The transition from cell division to cellular expansion in *Arabidopsis* leaves starts at the distal part and ends at the pedicel (Andriankaja *et al.*, 2012). This transition happens very quickly and is highly coordinated.

6.2. Control of cell division

It is important to make a distinction between cellular growth and cellular expansion, because proliferating cells need to grow before dividing. This growth is an increase in the cytoplasmic mass. In contrast, the cellular expansion is based on an increase in cellular volume by vacuolation (Sugimoto-Shirasu and Roberts, 2003).

The triggering of cell proliferation in *Arabidopsis* includes a cascade of genes downstream of the phytohormone auxin. This cascade starts with *ARGOS* (Hu *et al.*, 2006). Sense or antisense constructs in transgenic plants cause elongation or reduction of lateral organs. *ARGOS* upregulates *AINTEGUMENTA* (*ANT*), a gene that affects the period of cell proliferation (Mizukami and Fischer, 2000). Another gene promoting cell division is *GROWTH REGULATION FACTOR 5* from *Arabidopsis* (*AtGRF5*), a putative transcription factor, whose overexpression results in the induction and/or maintenance of cell proliferation activity in leaf priomordia. Similar effects has *ANGUSTIFOLIA3/GRF-INTERACTING FACTOR1 (AN3/GIF1)*, a homologue of the human transcriptional coactivator *SYNOVIAL SARCOMA TRANSLOCATION* (*SYT)*, that interacts with *AtGRF* (Horiguchi *et al.*, 2005). Inducing cell division in an organ is regulated by *JAGGED (JAG)*, a putative C_2H_2 zinc-finger transcription factor, and *NUBBIN* (*NUB*), a gene closely related to *JAG*, and acting in a redundant way in growing areas of lateral organs in *Arabidopsis*. However, another mechanism to promote cell division is controlled by the gene *KLUH* (*KLU*) from *Arabidopsis*, a P450 cytochrome. Loss-of-function mutants of *klu* show a premature stop in cell division and smaller organs, whereas overexpression of *KLU* produces larger organs with more cells. (Anastasiou *et al.*, 2007; Kazama *et al.*, 2010). Gene products that limit cell division usually are not uniformly distributed over organs or organ parts, for example stopping of cell division in leaves starts at the tip and involves TCP class II genes as *CINCINNATA* (*CIN*) of *Antirrhinum*, whose loss of function leads to prolonged proliferation in the leaf margin (Nath *et al.*, 2003). The double mutant of *Arabidopsis PEAPOD 1* and *2* shows an increment in the internal division of the leaf margin, which gives bell shaped leaves (White, 2006).

The miR396 miRNA antagonizes the expression pattern of transcription factor *GRF* and preferably is accumulated in the distal parts of the leaf by the attenuation of cell division (Rodriguez *et al.*, 2010). The dominant mutant *GRANDIFLORA* (*GRAF*) of *Antirrhinum* shows an increment in cell division especially in stamens and style, indicating that *GRAF* limits cell division in an organ specific manner (Delgado-Benarroch, *et al.*, 2009). Finally the *FORMOSA* (*FO*) gene of *Antirrhinum* specifically regulates flower size by inhibiting cell division and this inhibition is due to a downregulation of *AmANT* (Delgado-Benarroch, *et al.*, 2009). A more general action upon size by restricting cell proliferation is conferred by the gene *DA1* (DA means 'large' in Chinese) in *Arabidopsis*. This gene

encodes an ubiquitin receptor and affects seed and organ size in parallel with *BIG BROTHER* (*BB)* and its allelic gene Enhancer of *DA1* (*EOD1)*. The expression of *DA1* is induced by the growth regulator abscisic acid (ABA), establishing a connection between ABA and limiting the proliferative growth during organogenesis (Li *et al.*, 2008).

Although we might conclude that the control of cell proliferation seems to be well preserved, this is not translated into a uniform pattern through a specific organ.

6.3. Control of cell expansion

The genetic control of cell expansion can also be divided into promoting and limiting factors. A promoting factor is the level of ploidy. Although the exact mechanism is not known yet, defects in the separation of chromatin based on double mutations that affect topoisomerase VI (topo VI) restricts the endoreduplication to two cycles and decreases cell growth (Sugimoto-Shirasu *et al.*, 2002).

The expansin superfamily is a group of genes involved in cell wall loosening through their effect on synthesis or on cellulose deposition. The decreased expression of the *expansin* gene *PhEXP1α* of *Petunia hybrida* causes smaller cells and reduces the size of the petals (Zenoni *et al.*, 2004). Members of two families of genes that stimulate both proliferation and cell enlargement have been found, which indicates a common regulatory mechanism for these two processes. The gene family of *Arabidopsis GRF*, which comprises nine members, includes the stimulator of cell division *AtGRF5*, while *AtGRF1*, *2* and *3* affect the expansion, as shown by decreased cell size in leaf tissue and cotyledons of the triple mutant (Kim *et al.*, 2003). Similarly, while *ARGOS* promotes cell proliferation, overexpression of *ARGOSLIKE* (*ARL*), a gene suggested to partially mediate brassinosteroid signalling and promoting cell expansion during organ growth, produces larger organs due to larger cells (Hu *et al.*, 2006). These examples show that a particular gene or a gene family can promote both cell division and cell expansion. In addition, action on cell division or cell expansion of a particular gene can be organ type specific or organ region specific. The mutant *Grandiflora* (*Graf*) from *Antirrhinum* produces larger cells in petals and increases cell division in stamens and styles, providing one of the few links between genes that control the size of the organ and organ identity (Delgado-Benarroch, *et al.*, 2009). The *FORMOSA* (*FO*) gene of *Antirrhinum* is a specific regulator of floral size in an organ region specific way, either as an inhibitor of cell division in the whole flower or as activator of cell expansion in conical cells of petals and the style (Delgado-Benarroch, *et al.*, 2009). The *COMPACTA ÄHNLICH* gene of *Antirrhinum* shows a promotion of cell expansion specific to an organ region. Mutations in this gene affect cell expansion in the proximal and distal regions of the petal, which

leads to a petal size reduction. This suggests a differential regulation between petal lobe regions (Delgado-Benarroch, *et al.*, 2009).

A limitation of cell expansion is also generated by *BIGPETAL*, a gene producing two mRNAs by alternative splicing, *BPEup* and *BPEp*. The first transcript appears in the whole plant while the second is preferably in petals. Lack of *BPEp* leads to bigger petals due to an increment in cell size (Szecsi *et al.*, 2006). Accumulation of *BPEp* mRNA is under the control of the identity of floral organs, providing a link between transcription pathways that regulate organ identity and are involved in growth resulting in a distinction between organ form and organ size. In accordance with this interpretation, a reduction in cell expansion in specific floral organs of the *Antirrhinum* mutant *formosa* coincides with a positive regulation of *AmBPE* (Delgado-Benarroch, *et al.*, 2009). The phytohormone jasmonate is required for controlling the expression of *BPEp,* thus *BPEp* is downstream of jasmonate in the signalling cascade during petal growth (Brioudes *et al.*, 2009). Summarizing, recent work in *Arabidopsis* proposes a cascade of events involved in the morphogenesis of the side organs where the meristem identity is regulated by a signal from cytokinins. That signal decays during the formation of lateral primordia, increasing auxin signal. This is replaced by a gibberellin signal that leads to processes controlled by methyl jasmonate (Reeves *et al.*, 2012; Nagpal *et al.*, 2005; Rubio-Somoza and Weigel, 2013). These routes appear to be coordinated by at least three miRNAs (Rubio-Somoza and Weigel, 2013). The miR167 controls *ARF6*/*ARF8* genes involved in auxin signalling (Wu *et al.*, 2006). The miR159 is regulated by gibberellins (Achard *et al.*, 2004). The loss of function in miR159a and b, two micro RNAs that target *MYB33* and *MYB65*, show developmental defects (Allen *et al.*, 2007). The miR319a is required for proper development of the petal and it targets *TCP4*, a member of the *TCP* transcription factor family which is called so because of the first characterized members (*TEOSINTE BRANCHED 1* (*TB1*), *CYCLOIDEA* (*CYC*) and *PROLIFERATING CELL FACTORS 1 and 2* (*PCFs*) (Cubas *et al.*, 1999), for degradation (Nag *et al.*, 2009).

Finally the *AtE2Ff* gene, an atypical member of the *E2F* transcription factors family, among others, affects cell size by controlling cell cycle transitions in an organ-specific manner. In the *e2ff*-1 mutant, hypocotyls are larger than wild-type, while overexpression leads to a reduction of hypocotyls due to changes in cell expansion. Since several genes involved in cell wall biosynthesis are direct targets of *AtEF2f*, it has been suggested that this gene may limit cell growth through direct suppression of cell wall biosynthesis (Fru *et al.*, 2004)

7. Genetic Analysis of Developmental Processes

Mutant plants are described as plants that have at least one variation in their DNA respect to the wild-type line, and show a phenotype for a given character. This variation may lead to a complete lack of gene functioning. The mutations can be classified into:

- Conditional mutation: mutation that depend on the environmental conditions.
- Deleterious mutation: affects reproduction capacity of the individual.
- Lethal mutation: causes unviable organisms at a certain stage.
- Morphological mutation: affects the morphology of the individual.
- Loss of function mutation: reduces the function of a gene, and in most cases show recessive segregations.
- Gain of function mutation: on rare occasions a mutation can lead to overexpression of a gene and/or ectopic expression. These alleles tend to show dominant segregation. .

The analysis of mutant plants begins with phenotypic studies and segregation analysis in F2 populations resulting from crosses between the mutant and the wild-type.

Relating a mutant phenotype to a specific genotype includes methods belonging to forward and reverse genetics (see section 2 for methods in reverse genetics) such as site-directed mutagenesis, gene silencing by RNA interference or gain-of-function, with the purpose to cause null or amorph (total loss-of-function gene), hypomorphic (partial loss-of-function gene), neomorphic (causes a dominant gain of gene function that is different from the normal function) or hypermorphic (gain-of-function) phenotype. Once the gene related to a specific mutant phenotype has been identified, its relationship to other non-allelic genes that might interfere with the observed phenotype, as well as the order of gene function within a functional pathway needs to be investigated. Epistatic gene interactions occur if a gene (epistatic) inhibits the expression of another gene (hypostatic) from a different locus. Several types of epistasis exist, including single-recessive epistasis, if a recessive allele blocks the expression of another gene, single-dominant epistasis, if the dominant allele prevents the expression of the hypostatic gene, double-recessive epistasis, produced by the double action of recessive alleles over any other allele, double-dominant epistasis, a case of gene redundancy, when the presence of at least a dominant allele masks the expression of another gene, and double dominant recessive, if the dominant allele of a locus and the recessive of another one respectively suppresses the action of the other alleles.

It has to be considered that, when two or more redundant genes encode the same protein, loss-of-function mutants do not show the desired reduction of gene expression. One of the reasons why genetic redundancy could be maintained by selection may be due to pleiotropic actions of the involved genes (Vavouri *et al.*, 2008).

In order to know in depth the interactions between genes and the order of gene function, double mutants are generated including those with loss- or gain-of-function of the target genes. There have been many studies using this technique over the years in yeast (Game and Cox, 1972), mice (Murphy *et al.*, 2003), rice (Lin *et al.*, 2000), tomato (Kachanovsky *et al.*, 2012) and human (Papaleo *et al.*, 2014).

8. Plant Transformation

The ability to regenerate plants in cell culture, coupled with the unique characteristics of the natural process of tumorigenesis induced by *Agrobacterium* species, laid the groundwork for the development of a whole methodology aimed at obtaining transgenic plants, in which the works done by the groups of Jeff Schell (Max-Planck Institute, Colonia) and Van Montagu (Universidad de Gante) have been decisive (Schell *et al.*, 1979; Hernalsteens *et al.*, 1980; Herrera-Estrella *et al.*, 1983). Ti plasmid attenuation by eliminating oncogenic T-DNA genes, and the replacement of these by the gene transfer, led to the development of binary vectors of suitable size which are used in this natural transformation system. The system also requires a promoter recognizable by DNA polymerase; it is common to use a constitutive promoter such as CaMV 35S promoter, in which characterization the Nam-Hai Chua group played a fundamental role (Benfey and Chua, 1990). Isolation of tissue-specific promoters which allow expression of the transgene in the appropriate time and place has become an important and economically interesting task, as the future of transgenic plants lies in utilizing promoters that show an activity in the situation and the tissue that the breeder needs.

Transferring genes by *Agrobacterium* bacteria is routinely performed in vegetables such as tomato, melon, cucumber and watermelon as well as in other important crop species such as corn, soybean, cotton and sunflower. Species of interest such as cereals or legumes however are difficult to transform through this system, although the problem appears to lie in the process of plant regeneration rather than in the transformation process. That is why alternative strategies have been developed to introduce DNA directly into the cells, either through microinjection using microprojectiles coated with the DNA for DNA transfer or by direct transformation of tissues in transient expression assays (Xiong *et al.*, 2013; Davies *et al.*, 2013; Wefers *et al.*, 2013).

It is worthwhile commenting on the leading role that reverse genetics has had in plant genetics from the mid-80s onward, since the existence of many enzymes and well characterized proteins enabled the isolation of the corresponding cDNA and the development of loss and gain of function experiments by antisense expression, co-suppression and ectopic expression. This led to the identification of genes whose potential for agriculture was unknown a priori. To this the development of the RNA interference technique, a new powerful molecular tool that allows to silence genes at the post-transcriptional level and identify functions associated with these genes, must also be mentioned. The RNA interference strategy is based on the degradation of messenger RNA by introducing a small homologous double chain RNA (siRNA) to the cellular messenger RNA of interest, causing the degradation of a sequence in a specific manner. Craig C. Mello and Andrew Fire received the Nobel Prize in Physiology or Medicine in 2006 for their discovery of siRNA (Grishok *et al.*, 2001).

Genetic transformation of plants and the cultivation of genetically modified varieties of tomato, corn, soybeans, cotton, rice, oilseed rape, sugar beet and alfalfa also has attracted excessive controversy. The scientific achievements, including herbicide resistance obtained by transfer of genes whose products detoxify the herbicide or block the receptor on which it acts, must be mention in this section. In the field of plant pathology, the initial interest was captured by transgenic crops expressing the protein *Bti* from *Bacillus thurigiensis var*. *israeliensis* whose toxicity and accumulation in the digestive tract of the insects causes death, and therefore the resistance of plants. More recently plant resistance to certain viruses has been achieved. The process by which transgenic plants expressing viral capsid proteins achieve immunity is still not well understood (Mason *et al.*, 1996).

Some other characters worth of mentioning in this section are those related to the quality of agricultural products. The molecular characterization of genes involved in fruit ripening process has led to the production of transgenic varieties called 'long life', especially in tomato. Here transgenesis prevents degradation of the fruit wall through strategies provoking antisense expression which can inhibit the biosynthesis of pectins and polygalacturonic acid, main components of the cell wall, or enzymes of the ethylene biosynthetic pathway, a hormone triggering the maturation process.

9. Problems Associated with Transformation

Handling of different microbial strains in the laboratory can turn into a problem when different bacteria become contaminated with each other. In most cases, contamination of bacterial strains is the result of sharing plasticware, pipettes and solutions. Furthermore, although different bacterial

genera can be grown on specific media, simplicity has led to a generalized use of common growth media as long as they do not interfere with bacterial properties. Two types of bacterial genera are routinely used in plant biology laboratories, *Escherichia coli* used for general cloning and *Agrobacterium tumefaciens* to obtain transgenic plants. But one obvious problem is that if *Agrobacterium* strains are contaminated with *E. coli*, then some of the processed colonies will not transform plants giving as result a very low or null transformation efficiency.

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Aims of the Thesis

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Chapter 1

The aim of this chapter was to develop a screening method based on DNA melting profiles for the detection of *Escherichia coli* contamination in *Agrobacterium tumefaciens* cultures as prerequisite for efficient plant genetic transformation.

Chapter 2

The goal of this chapter was to establish a stable protocol of *Antirrhinum majus* transformation previous to determine the quantitative requirements for floral organ identity genes during late flower development.

Chapter 3

The protocol of stable plant transformation developed in Chapter 2 was used in order to evaluate the possibility to modify floral organ size in model/ornamental species by variation of *AINTEGUMENTA* gene expression, a gene known to affect floral organ size in *Arabidopsis*.

Chapter 1

Chapter 1: Using 23S rDNA to identify contaminations of *Escherichia coli* in *Agrobacterium tumefaciens* cultures

Manchado-Rojo, María[a,b], Weiss, Julia[a,b] and Egea-Cortines, Marcos[a,b]*

[a] Departamento de Ciencia y Tecnología Agraria, Universidad Politécnica de Cartagena, 30203 Cartagena, Spain

[b] Instituto de Biotecnología Vegetal, Universidad Politécnica de Cartagena, 30203 Cartagena, Spain

*Corresponding Author: marcos.egea@upct.es (Marcos Egea-Cortines)
Telf: +34 968325705
Fax: +34 968325433

Abbreviations used: qPCR, quantitative PCR; VIGS, virus-induced gene silencing; SDS, sodium dodecyl sulphate; LB: Luria Bertani broth

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Abstract

Cross contamination of *Agrobacterium tumefaciens* stocks with *Escherichia coli* are difficult to identify by microbiological techniques, leading to false negative results in transformation experiments. We have developed a genotyping assay for *A. tumefaciens* and *E. coli* lab strains based on amplification of 23S rDNA by PCR. *Agrobacterium* strains LBA 4404, C58 and EHA105 and *E. coli* strains DB3.1, DH5α and XL1-Blue can be identified separating the corresponding PCR amplicons in 2.5% agarose gels. However, in crossed contaminations, interpretation of results is improved using melting point analysis on a quantitative PCR machine.

Handling of different microbial strains in laboratory practice can turn into a problem when different bacteria become contaminated with each other. In most cases, contamination of bacterial strains is the result of sharing plasticware, pipettes and solutions. Furthermore, although different bacterial genera can be grown on specific media, simplicity has led to a generalized use of common growth media as long as they do not interfere with bacterial properties. Two types of bacterial genera are routinely used in plant biology laboratories, *Escherichia coli* used for general cloning and *Agrobacterium tumefaciens* to obtain transgenic plants.

Recent advances in cloning strategies based on recombination cloning coupled with virus-induced gene silencing (VIGS) [1] allow research projects where hundreds to thousands of genes can be tested for loss of function. The standard procedure is the construction of cDNA libraries and development of recombinant libraries in vectors suitable for VIGS. These recombinant libraries are then transferred to *Agrobacterium* that is used to obtain transiently transformed plants expressing the cDNA cloned as double stranded RNA that causes post transcriptional gene silencing. This technology has been shown to work in different plants like *Papaver* [2], potato [3] or *Petunia* [4].

But one obvious problem is that if *Agrobacterium* strains are contaminated with *E. coli*, then some of the colonies processed will not transform plants giving as a result a false negative. Although *E. coli* and *Agrobacterium* can be differentiated by microbiological techniques, it is much faster to establish a genotyping protocol based on PCR that will allow the identification of *E.coli* contaminations in those cases where there is doubt of the purity of the *Agrobacterium* strain. In this study we report the development of a genotyping assay to distinguish between *E. coli* and *Agrobacterium*.

We grew on LB medium to complete saturation the *E. coli* strains DH5α, and DB3.1 from Invitrogen and XL1-Blue from Stratagene at 37ºC while the *Agrobacterium* strains LBA 4404, C58 and EHA105

were grown at 28ºC. Extraction of bacterial genomic DNA was performed starting from 1.5mL of saturated culture. Samples were centrifuged and the bacterial pellets were resuspended in 1 mL of TE buffer (10 mM Tris HCL, 1mM EDTA, pH 8.0) and lysed with 200 µg of lysozyme and 1% SDS. Protein was digested with 500 µg of proteinase K incubating at 65ºC for one hour. Genomic DNA was purified from the samples by standard phenol:chloroform extraction and precipitation [5]. The resulting pellets were resuspended in 100 µL of TE buffer. Amongst the possible genome regions valuable for genotyping, the intergenic transcribed spacer (ITS) has been found useful in plants [6], the 23S rDNA and the 16S-23S rDNA spacer in bacteria [7], or 5.8S-ITS region to identify yeast contamination in food samples [8]. We used specific primers that amplify the bacterial 23S rDNA (23S-forward ACCAGGATTTTGGCTTAGAAG and 23S-reverse CACTTACCCCGACAAGGAAT) [7] in a standard PCR experiment performed with Taq polymerase from Promega. The cycling conditions consisted in thirty cycles of 30 seconds at 94ºC, 30 seconds at 62ºC and 30 seconds at 72ºC. Reaction products were separated using 2.5% agarose gels containing ethydium bromide, and visualized with a UV transilluminator. Amplification products from *Agrobacterium* and *E. coli* could be visually differentiated on the gel, showing PCR fragments of 780 and 850bp respectively (Fig.1A). This suggests that direct analysis of PCR products is feasible. However, we found that in complex mixtures of bacterial strains i.e when there is contamination of *Agrobacterium* with *E. coli*, it would be better to have a more powerful assay since the size differences between amplicons make results difficult to interpret on agarose gels (data not shown). We solved this problem by performing a RT-qPCR on a CR-Corbett Research-quantitative PCR using TaKara Sybr Green quantitative PCR amplification kit. The PCR conditions were an initial denaturation of 5 minutes at 95ºC followed by forty cycles with a scheme of 95ºC 30 seconds, 62ºC 30 seconds, 72ºC 30 seconds, a read at 83ºC for 15 seconds and finally a melting point analysis starting at 60ºC and reaching 94ºC with reads every 0.5ºC for 15 seconds. The melting point of the 23S rDNA region of *Agrobacterium* and *E. coli* differed by 1.6ºC (87.9 and 89.5 respectively), giving clear-cut results that allow the genotyping of *Agrobacterium* and *E. coli* (Fig 1.B).

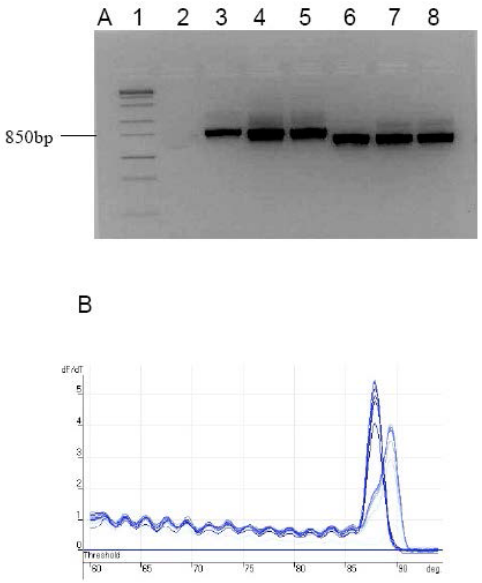

Figure 1: (A) Amplification of 23S rDNA from bacterial samples by PCR. Lane 1 molecular weight; Lane 2 negative control; Lane 3 *E. coli* DB3.1; Lane 4 *E. coli* DH5α; Lane 5 XL1-Blue; Lane 6 *A .tumefaciens* LBA4404; Lane 7 C58; Lane 8 EHA105. Molecular weight markers were ZipRuler ladder-1 from Fermentas with molecular weights of 10000, 5000, 3000, 2000, 1200, 850, 500, 300 and 100 bp. **(B)** Melting curve of samples of *A. tumefaciens* and *E. coli*. The peak at 89.5 degrees corresponds to duplicated samples of DB3.1, DH5α and XL1-Blue, while the peak at 87.9 corresponds to LBA4404, C58 and EHA105 *A. tumefaciens* strains.

In summary, we developed a PCR assay to genotype *Agrobacterium* and *E. coli* that helps overcome situations of cross contamination between these types of bacteria. The protocol presented should be useful to test clones that do not appear to transform plants, and this lack of transformation capacity could be the result of contaminated bacterial stocks. Our approach has the additional advantage of being based on codominant PCR markers that should be more useful than *Agrobacterium* or *E. coli* specific PCR makers.

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Chapter 2

Chapter 2: Quantitative levels of *Deficiens* and *Globosa* during late petal development show a complex transcriptional network topology of B function

María Manchado-Rojo, Luciana Delgado-Benarroch[1], María José Roca[2], Julia Weiss and Marcos Egea-Cortines*

Genetics, ETSIA, Instituto de Biotecnología Vegetal, Universidad Politécnica de Cartagena, Paseo Alfonso XIII 48, 30203 Cartagena, Spain.

[1] Present Address: Instituto de Botánica del Nordeste (IBONE-CONICET), Facultad de Ciencias Agrarias, Universidad Nacional del Nordeste. Sargento Cabral 2131 CC 209, (3400) Corrientes, Argentina.

[2] Servicio de Apoyo a la Investigación Tecnológica, Universidad Politécnica de Cartagena, Plaza del Hospital s/n, 30202 Cartagena, Spain

* Corresponding Author: Genetics, ETSIA, Universidad Politécnica de Cartagena, Paseo Alfonso XIII 48, 30203 Cartagena, Spain.
Telephone: 34868071077; Fax: 34968325433; e-mail: marcos.egea@upct.es

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Summary

The transcriptional network topology of B function in *Antirrhinum*, required for petal and stamen development, is thought to rely on initial activation of transcription of *DEFICIENS* (*DEF*) and *GLOBOSA* (*GLO*), followed by a positive autoregulatory loop maintaining gene expression levels. Here, we show that the mutant *compacta* (*co*), whose vegetative growth and petal size are affected, plays a role in B function. Late events in petal morphogenesis such as development of conical cell area and scent emissions were reduced in *co* and *def*^{nicotianoides} (*def*^{nic}), and absent in *co def*^{nic} double mutants, suggesting a role for *CO* in petal identity. Expression of *DEF* was down-regulated in *co* but surprisingly *GLO* was not affected. We investigated the levels of *DEF* and *GLO* at late stages of petal development in the *co*, *def*^{nic} and *glo-1* mutants, and established a reliable transformation protocol that yielded RNAi-*DEF* lines. We show that the threshold levels of *DEF* or *GLO* required to obtain petal tissue are approximately 11% of wild-type. The relationship between *DEF* and *GLO* transcripts is not equal or constant and changes during development. Furthermore, down-regulation of *DEF* or *GLO* does not cause parallel down-regulation of the partner. Our results demonstrate that, at late stages of petal development, the B function transcriptional network topology is not based on positive autoregulation, and has additional components of transcriptional maintenance. Our results suggest changes in network topology that may allow changes in protein complexes that would explain the fact that not all petal traits appear early in development.

Introduction

Seminal work in *Antirrhinum majus* and *Arabidopsis thaliana* allowed formulation of a combinatorial model based on gene functions explaining what later was found to be a general scheme of floral organ development in angiosperms (Schwarz-Sommer *et al.*, 1990; Coen and Meyerowitz, 1991; Egea Gutierrez-Cortines and Davies, 2000; Causier *et al.*, 2010). The so-called ABC model has been tested and interpreted in various forms in several plant species. B function genes are involved in petal and stamen morphogenesis, and two genes, *DEFICIENS* and *GLOBOSA*, perform this task in *Antirrhinum* (Sommer *et al.*, 1990; Trobner *et al.*, 1992).

Petal development involves several subsets of genes activated by the B function. Amongst the features of a fully mature petal are the distinct colours displayed, resulting from pigment synthesis and down-regulation of chlorophyll production, proper size and shape, and release of scent. Petal development is not a linear process. The phenylpropanoid synthesis pathway involved in petal pigmentation does not show simple activation, but instead follows a pattern of early and late gene expression (Martin and Gerats, 1993). This is true in *Antirrhinum* and *Petunia*, indicating that some

fine regulatory aspects of petal development may be conserved in evolution (Almeida *et al.*, 1989; Martin *et al.*, 1991; Weiss *et al.*, 1993). Petal growth also displays bi-phasic behaviour in *Petunia*, *Gerbera* and *Arabidopsis*, with petal development promoted by cell division and later stages promoted by cell expansion (Reale *et al.*, 2002; Anastasiou and Lenhard, 2007; Laitinen *et al.*, 2007). In *Antirrhinum*, petal growth is somewhat more complex, as cell division occurs in early stages and regions where cell division occur are also detected late in development (Perez-Rodriguez *et al.*, 2005; Delgado-Benarroch *et al.*, 2009a).

At later stages, petal epidermal cells become conical as a result of expression of the *MIXTA* gene (Noda *et al.*, 1994). This process is conserved among species, and both conical cell development and the angle of petal reflection are controlled partly by *MIXTA* in *Antirrhinum* and *Petunia* (Baumann *et al.*, 2007). Conical cell formation in *Antirrhinum* continues until late stages of petal development, well after anthesis and before petal abscission (Goodwin *et al.*, 2003). *MIXTA* and *MIXTA-LIKE* genes have a conserved function in conical cell development during evolution (Di Stilio *et al.*, 2009). *MIXTA* expression is known to be controlled by B function genes, as plants expressing unstable alleles of *def* have been shown to display conical cells in reverting sectors (Carpenter and Coen, 1990). The level of expression of *MIXTA* and *MIXTA-LIKE-1* depend on the quantitative levels of *DEF* and *GLO* in *Antirrhinum* (Perez-Rodriguez *et al.*, 2005). Conical cells have several biological functions related to pollinator attraction, including scent production (Kolosova *et al.*, 2001; Whitney *et al.*, 2009a,b, 2011). The complex floral scent profile of most plants is the result of unique blends of compounds, whose production is due to activation of several biochemical pathways (Vainstein *et al.*, 2001). The *Antirrhinum* scent profile includes methyl benzoate, a product of the phenylpropanoid synthesis pathway, and terpenoids such as myrcene and ocimene (Dudareva *et al.*, 2000, 2003).

Activation of floral homeotic genes in *Arabidopsis* requires two partially redundant paralogs, *APETALA1* (*AP1*) and *CAULIFLOWER* (Kempin *et al.*, 1995; Ferrandiz *et al.*, 2000). Negative regulation of the genes *AGAMOUS-LIKE24* (*AGL24*) and *SHORT VEGETATIVE PHASE* (*SVP*) by *AP1* is required to activate *SEPALLATA* (*SEP*) (Kaufmann *et al.*, 2010b). The *SEP1–4* family is important to activate B function in *Petunia*, tomato and *Arabidopsis* (Angenent *et al.*, 1993; Pnueli *et al.*, 1994; Pelaz *et al.*, 2000; Vandenbussche *et al.*, 2003). The resulting B function activity is maintained by a positive autoregulatory loop that has been described in *Antirrhinum*, *Petunia* and *Arabidopsis* (Schwarz-Sommer *et al.*, 1992; Halfter *et al.*, 1994; Zachgo *et al.*, 1995; Honma and Goto, 2000; Vandenbussche *et al.*, 2004). The B function gene products form heterodimers (Davies *et al.*, 1996b; Winter *et al.*, 2002; Wang *et al.*, 2010) that can bind their own promoters and activate transcription. Plants expressing hypomorphic alleles of *def* display progressively smaller petals that become more

sepalloid with the strength of the alleles (Schwarz-Sommer *et al.*, 1992; Zachgo *et al.*, 1995; Bey *et al.*, 2004). Petal growth as a whole is affected in B function homeotic mutants. Plants expressing null *def*^{globifera} (*def*^{gli}) and *glo-1* alleles display second-whorl organs that are indistinguishable from first-whorl sepals (Trobner *et al.*, 1992), and sepals are much shorter than petals in *Antirrhinum majus* (Delgado-Benarroch *et al.*, 2009a). As a result of the studies described above and others performed in *Arabidopsis* (Szecsi *et al.*, 2006; Kaufmann *et al.*, 2009), there is a general agreement that floral organ size requires proper function of the floral organ identity genes (Yu *et al.*, 2004; Dornelas *et al.*, 2011).

Although positive autoregulatory loops occur as network motifs in many biological pathways (Heintzen *et al.*, 1997; Varghese and Cohen, 2007; Fujiwara *et al.*, 2009), they are inherently slow to respond to variation of gene expression (Kalir *et al.*, 2005; Alon, 2007), which may result in decreased flexibility (Ma *et al.*, 2006). Given the importance of the B function in terms of petal and stamen identity, it is possible that B function transcriptional maintenance includes gene activation, positive autoregulatory loop control and some parallel or ancillary components that add robustness to the system. Indeed, the obligate heterodimerization of canonical B function gene products adds robustness to the system at the post-transcriptional level (Espinosa-Soto *et al.*, 2004; Lenser *et al.*, 2009; Kaufmann*et al.*, 2010a; Geuten *et al.*, 2011). Further robustness is probably achieved as a result of larger MADS box complexes, which may aid in stabilization of the protein–DNA binding complexes (Egea-Cortine *e al.*, 1999; Theissen and Saedler, 2001; Melzer and Theissen, 2009; Kaufmann *et al.*, 2010a). Despite these protein stabilization processes, decreases in B function gene expression cause clear homeotic changes in *Antirrhinum*, *Petunia* (Vandenbussche *et al.*, 2004; Rijpkema *et al.*, 2006) and *Arabidopsis* (Irish and Yamamoto, 1995), demonstrating the importance of sustained transcriptional activity of the B function.

Although the initial steps of petal development are understood in some detail, late stages of development are thought to be a follow-up, but, to the best of our knowledge, no quantitative analysis of B function transcription at late stages of petal development has been performed to support this hypothesis.

Here we report genetic characterization of the mutant *compacta*, a classic *Antirrhinum majus* mutant (Kuckuck and Schick, 1930) in which leaf shape and floral size are affected. We uncover a genetic interaction of *co* with *def* that implicates *CO* in activation of *DEF* expression. We measured scent production in the *co* and *def*^{nic} mutants, and found that production of methyl benzoate, ocimene and myrcene was reduced in *co* and *def*^{nic} mutants and was completely absent in *co def*^{nic} double

mutants, demonstrating a role for *co* in B function. In order to establish the threshold of B function, we developed a highly reliable transformation protocol and obtained *RNAi-DEF* lines. We performed quantitative expression analysis of *DEF*, *GLO* and *MIXTA* in *co def*^{nic} double mutants, *glo-1* and *RNAi-DEF* lines. We identified the thresholds of *DEF* and *GLO* associated with different levels of petal identity, and surprisingly found that the levels of *DEF* and *GLO* transcripts changed at later stages of development, which was unexpected from a network topology based exclusively on positive autoregulation. We propose a model of B function transcriptional control that accommodates the data presented and may explain petal development as a multistep process.

Results

The *co* mutation affects petal and stamen cell size

The mutant *compacta* had been described as a recessive mutant (Stubbe, 1966), and segregation analysis of a cross of *co* with the laboratory wild-type line 165E confirmed this result (Schwarz-Sommer *et al.*, 2010). As previously described, we found that the *co* mutation affected vegetative growth, including decreased internode elongation and smaller leaves that were altered both in width and length (Table 1). Leaf number until the first flower was not different from wild-type, indicating that the *co* mutation does not affect floral transition.

Table 1: Comparison of vegetative parameters between wild-type and the *co* mutant

Genot.	Internode (mm)			Leaf 1 (mm)		Leaf 2 (mm)		Leaf 3 (mm)		Leaf number		
	1	2	3	Length	Width	Length	Width	Length	Width	Decusate	Spiral	Total
co	10.4±3.4	16.8±7.4	21±4.9	16.1±2.2	11.2±1.2	25 9±1.5	15.6±1 0	35.5±5 9	16±2.4	8.8±1.2	5.2±4.1	14±4.0
Wt	18 9±1.9	27.2±3.2	31.8±4 3	24.1±2.1	13.4±0.8	39±2.9	19.4±1.6	46.1±4 9	18 9±2.9	8.1±1.3	3.7±2.7	11 8±1.8
%	-45.16***	-38.41***	-33.89***	-33.22***	-16.39***	-33.57***	-19.36***	-23.01***	-15.48*	8.64	40.54	15.71

Values of internode size and leaves are means ± standard deviation (n = 15). Percentages refer to wild-type siblings in the F_2 segregating population. Asterisks indicate significant differences between the *co* mutant and wild-type: *P < 0.05; **P < 0.01; ***P < 0.001.

The flowers of *co* plants may be easily distinguished from those of wild-type because stamens protrude outside the tube (Figure 1a). Furthermore, they were significantly smaller than those of wild-type (Figure 1b, 1c and Table 2) for all floral parameters analyzed, except for the adaxial stamens, which retained wild-type size (Figure 1d). In many cases, under greenhouse conditions, the adaxial protruding stamens tended to show dehiscence and dehydration before the flowers were fully open. Furthermore, the abaxial stamens appeared more separate than in wild-type, forming a characteristic V shape (Figure 1d). Hand self-pollinated *co* flowers were fully fertile. Flower colour was not affected in *co* mutants, which displayed colour segregation of the *nivea*, *delila* and *pallida recurrens* loci (Figure S1) present in the 165E and Sippe50 wild-type background

Figure 1. Wild-type (left) and *co* mutant flowers (right) from the side, and longitudinal sections.

Table 2. Comparison of floral parameters for wild-type, the *co* mutant, the *def*^{nic} single mutant and the *co def*^{nic} double mutant

Genotype	Tube length (mm)	Lower length (mm)	Petal height (mm)	Sepal length (mm)	Tube width (mm)	Upper (total) length (mm)	Lower petal expansion (mm)	Upper petal expansion (mm)	Stamen length (mm)	Gynoecium length (mm)
Wt	17.9±0.7	30.8±2.7	26.4±2.7	7.9±0.8	12.1±0.8	39.5±0.9	26.2±1.6	29.9±2.0	25.6±0.9	22±0.7
co	15.3±0.8	23.5±1.3	17.1±2.3	6.6±0.5	10.4±0.7	28.3±1.4	19.2±2.7	16.4±2.3	26±1.8	20.1±1.0
def^{nic}	8.7±4.9	20.5±2.0	11.2±1.9	6.4±0.6	7.1±0.5	20.8±2.3	15.8±2.9	11.7±2.3	16.6±1.3	19.3±2.3
co def^{nic}	5.3±0.5	11.4±1.7	8.1±1.0	7.2±0.8	5.7±0.5	12.0±1.9	7.6±1.5	8.1±0.8	14.8±0.6	16.9±0.9
% *def*^{nic} vs *co def*^{nic}	-38.38*	-44.64***	-27.93***	12.80	-19.26***	-42.25***	-51.82***	-30.37**	-10.62**	-12.17*
%*co* vs Wt	-15.07***	-23.64***	-35.01***	-16.48***	-14.03***	-28.37***	-26.93***	-45.09***	1.40	-8.68***

Total number of measurements for each parameter = 10. Values are means ± standard deviation. Asterisks indicate significant differences using the wild-type or *def*^{nic} single mutant as: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

We investigated the effects of the *co* mutation on cell division and expansion in the various floral organs. Sexual organs in the *co* mutant showed independent cellular phenotypes, as cells in stamens were significantly smaller than in wild-type, whereas cell size in styles was not affected (Figure 2 and Table 3). With regard to petals, flat cells proximal to the tube did not show significant differences with respect to wild-type flowers. However, in the distal part of the petal, conical cells were 43% smaller than in wild-type, and flat cells showed a decrease in area of 33%. Altogether, the observed petal phenotypes may be explained by a decrease in cellular size in the petals (Figure 2 and Table 3).

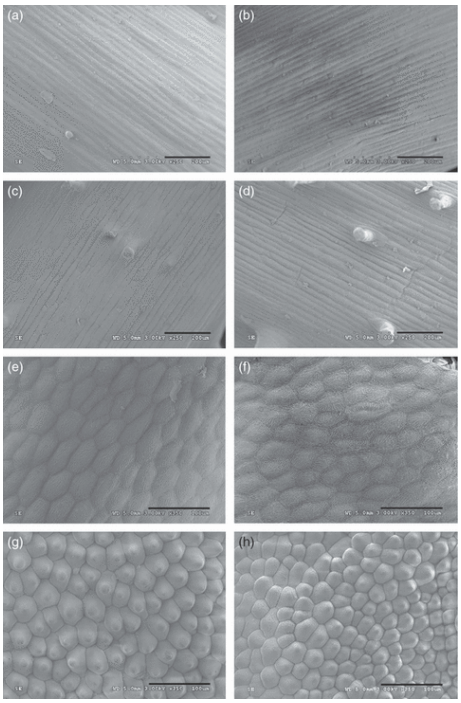

Figure 2. Scanning electron microscopy of cells from various organs of fully developed flowers of wild-type (left) and *co* mutant flowers (right). (a, b) Third-whorl styles, (c, d) fourth-whorl gynoecium, (e, f) dorsal petal, proximal to the tube, and (g, h) dorsal petal, distal part. Scale bars = 100 µm.

Table 3. Cell area of petal, stamen and style in wild-type and the *co* mutant

Genotype	Stamen (μm^2)	Style (μm^2)	Petal (μm^2)	
			Conical cells	Flat cells
co	2948.4±103.9	2123.1±85.1	756.9±29.0	1609.66±52.19
Wild-type	3568.1±76.4	2221.9±70.5	1346.5±43.3	2101.24±55.83
%	-17.37*	-4.45	-43.78*	-23.39*

Total number of cells measured for each organ/mutant = 50. Values are means ± standard error. Asterisks indicate significant differences between the *co* mutant and wild-type: *$P < 0.05$.

Co plays a role in B function

As petal and stamen cell size were significantly reduced in the *co* mutant, we investigated a possible interaction with organ identity. We crossed *co* with the weak allele *def*^{nic}. This allele affects second- and third-whorl organ identity, with sepaloid petals that are smaller than wild-type petals but still develop colour and conical cells (Schwarz-Sommer *et al.*, 1992). We constructed an F_2 population of *co* × *def*^{nic}, obtaining a Mendelian segregation of 47 wild-type, 17 *co*, 19 *def*^{nic} and seven plants with a stronger phenotype ($\chi^2 = 0.8642$, d.f. = 3, $P = 0.8341$). The plants with floral phenotypes differing from *co* or *def*^{nic} single mutants were considered *co def*^{nic} double mutants. The phenotype resembled *def*^{nic} but was more extreme, in some cases showing second-whorl sepalloid organs resembling those of *def*^{gli} null mutants (Figure 3a–c). We selfed *co* mutant siblings of the putative double mutants and obtained a segregation of 3:1 for plants displaying an enhanced *def*^{nic} phenotype, thus confirming that the effect of the *co* mutation is an enhancement of *def*^{nic}. The *CO* gene is not allelic to *DEF* based

on the F_1 phenotypes that were wild-type and the fact that these genes map to linkage groups 6 and 8, respectively (Schwarz-Sommer *et al.*, 2010).

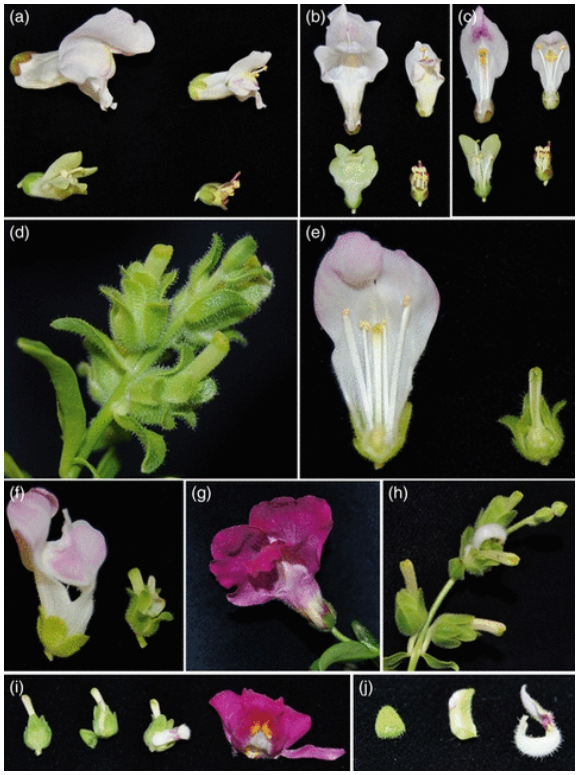

Figure 3. Phenotypes and lines. (a–c) Top left, wild-type; top right, the *co* mutant; bottom left, *def*^{nic}; bottom right, *co def*^{nic} double mutant. (d) *RNAi-DEF* transgenic line showing extreme phenotypes similar to the classic *def*^{gli} null allele. (e) Section of wild-type (left) and the *RNAi-DEF* strong phenotype line (right). (f) Wild-type (left) and partially reverting *RNAi-DEF* flower with chimeric second-whorl organ (right). (g) *RNAi-DEF* flower with wild-type appearance. (h) *glo-1* mutant showing a revertant second-whorl organ. (i) Comparison of *glo-1* flowers, showing two without reversion, one with a partial petaloid structure in the second whorl, and a petal with wild-type appearance from the *RNAi-DEF* line. (j) Close-up of increasingly wild-type second-whorl organs from *glo-1* flowers.

We compared organ size in *co* mutants and *co def*^{nic} double mutants, and found that all measured parameters were significantly smaller except first-whorl sepals (Table 2), indicating a synergistic effect of the *co* and *def*^{nic} mutations. The most prominent decreases in size in the second whorl corresponded to the tube length and lateral expansion of the abaxial petals, which showed mean reductions >60% ($P < 0.001$). Furthermore, *co def*^{nic} double mutants completely lacked the typical *Antirrhinum* petal palate. Modest but significant reductions in size were found in the third whorl, which showed partial carpelloid structures, although stamens were formed in all flowers analyzed.

We examined cellular morphologies and sizes in petals of *def*^{nic}. As previously described, *def*^{nic} mutants showed a range of cells reminiscent of wild-type sepal and petal cell types. We observed typical puzzle cells seen in sepals, with a gradient towards the distal portion of the petal that started with flat oval-shaped cells that increased gradually in size until true conical cells formed at the edges of the petaloid organs (Figure 4). Both the size of the conical cells and the overall surface of the petal

were greatly reduced compared to wild-type petals or *co*. In contrast, we did not find conical cells in *co def*^{nic} double mutant flowers, which displayed both puzzle cells and flat cells. Differences in cell morphology between the *co* mutant and the *co def*^{nic} double mutant were pronounced enough not to permit legitimate comparison of cell sizes. The lack of conical cells in *co def*^{nic} double mutants strongly suggests that *co* itself plays a role, not only in determination of cell size, but also in late petal development. Cells in the third whorl of *co def*^{nic} double mutants showed a decrease in size beyond that found for *def*^{nic} single mutants. The fourth-whorl style cells in wild-type and *co* were not significantly different (Table 3). However, the *co def*^{nic} double mutant had larger cells than *def* single mutants despite the fact that this organ is smaller (Table 4). This suggests that *co* may interact with other genes involved in carpel and stamen development such as *PLENA* and *FARINELLI* (Bradley *et al.*, 1993; Davies *et al.*, 1999).

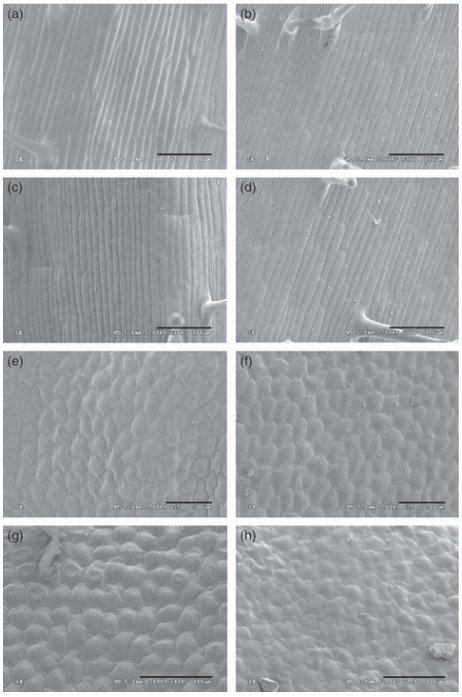

Figure 4. Scanning electron microscopy of floral organs of *def*^{nic} mutant (left) and *co def*^{nic} double mutant (right). (a, b) Third-whorl organs, (c, d) gynoecia, (e, f) dorsal petal proximal region of flat cells, and (g, h) dorsal petal distal region of conical cells. Scale bars = 100 µm.

Table 4. Cell area of stamen and style in the *def*^{nic} single mutant and the *co def*^{nic} double mutant

	Stamen filament (µm^2)	Style (µm^2)
Wild-type	3568.09±154.43	2221.99±70.49
def^{nic}	2213.4±96.5	1466.9±76.2
co def^{nic}	1940.5±76.6	1842.2±70.3
% *co* vs *wt*	-17.37***	-4.45
% *def*^{nic} vs. *co def*^{nic}	-12.33*	+25.58***

Total number of cells measured for each organ/mutant = 50. Values are means ± standard error. Asterisks indicate significant differences using the wild-type or *def*^{nic} single mutant as: *$P < 0.05$; ***$P < 0.001$.

Co plays a role in scent production

We measured the production of three major scent compounds (myrcene, ocimene and methyl benzoate) in fully developed flowers of a segregating population of *co* and *def*^{nic}, 24 h after anthesis (Table 5 and Figure S2). We observed emission of the three compounds in wild-type flowers. However, we did not find myrcene in any of the *co* samples analyzed, and the levels of emission of ocimene and methyl benzoate were similar to those of wild-type. In *def*^{nic} flowers, we found levels similar to wild-type for myrcene, whereas ocimene and methyl benzoate were drastically reduced. The phenotype of the *co def*^{nic} double mutant was extreme concerning scent emission, as we were not able to detect myrcene, ocimene or methyl benzoate in any of the samples analyzed.

Table 5. Effect of the *co* and *def*^{nic} mutations on volatile levels

Genotype	Myrcene	Ocimene	Methyl benzoate
Wild-type	40.2	251.2	137.2
co	ND	156.6	165.4
def^{nic}	34.4	9.7	29.6
co def^{nic}	ND	ND	ND

Quantities refer to mean emissions of three samples (ng g^{-1} tissue h^{-1}). ND indicates that we could not detect the compound in any sample.

Co is involved in *DEF* transcriptional control and plays a role in B function

As the *co* mutant showed a number of phenotypes that may be described as B function-related, we investigated the effect of the *co* mutation on the B function transcriptional network by quantitative gene expression analysis. We found that *DEF* gene expression was significantly down-regulated in *co* mutants to 11.6% of the value found in wild-type (P = 0.04; Figure 5a), similar to the down-regulation in *def*^{nic} (P = 0.653) versus wild-type. Surprisingly, the levels of *GLO* expression were higher in *co* than in wild-type, but not significantly (P = 0.23). This finding is contrary to what would be expected from a positive autoregulatory loop scheme (Schwarz-Sommer *et al.*, 1992), which suggests simultaneous down-regulation of *GLO* as observed in *def*^{nic} (P = 0.029). These unexpected results indicate that *CO* is involved in the activation or maintenance of *DEF* expression, whereas *GLO* is not directly affected. In the *co def*^{nic} double mutant, both *DEF* and *GLO* expression were down-regulated compared to wild-type. Furthermore, the high levels of *GLO* in the *co* mutant may be partly responsible for the better petal development observed compared to *def*^{nic}, but the results for the double mutant indicate that *CO* also plays a downstream role, beyond the uncovered effect on *DEF* expression, and may be considered a B function gene itself, supporting the previous data on cell types and scent emission.

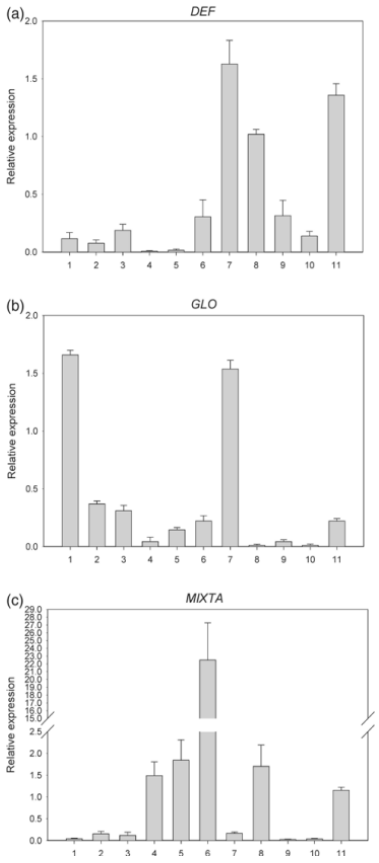

Figure 5. Relative expression of (a) *DEFICIENS*, (b) *GLOBOSA* and (c) *MIXTA* in second-whorl organs compared to wild-type petals. An arbitrary level of 1 was assigned to the wild-type: 1, the *co* mutant; 2, def^{nic}; 3, *co* def^{nic} double mutant; 4, *RNAi-DEF* sepal; 5, *RNAi-DEF* petal/sepal; 6, *RNAi-DEF* petal; 7, *RNAi-DEF* petal (normal flower); 8, *RNAi-DEF* tube; 9, *glo-1* sepal; 10, *glo-1* revertant; 11, *glo-1* petal.

Quantitative analysis of transcriptional regulatory network in late petal development

In order to obtain a comprehensive picture of gene expression levels of *DEF* and *GLO*, and identify the quantitative thresholds supporting different degrees of petal development, we used a mixture of genetic backgrounds. We developed an improved protocol to transform *Antirrhinum majus* (see Experimental procedures and Appendix S1), and obtained two independent transgenic lines harbouring an *RNAi-DEF* construct that were positive for kanamycin resistance and showed a range of phenotypes from weak to null *def* alleles (Figures 3d and S3). Flowers of the strongest line showed two whorls of sepals and a third whorl of carpels, typical of a def^{gli} allele (Figure 3e). In a direct comparison, they could not be distinguished from flowers expressing def^{gli} or *glo-1* null alleles. The strongest line showed progressive acropetal loss of the extreme phenotype, displaying flowers with second-whorl sepal/petal chimeric organs (Figure 3f), and eventually reverted completely to produce apparent wild-type flowers (Figure 3g).

We gathered second-whorl organs from the aforementioned single and double mutants and the strong *RNAi-DEF* line. In order to obtain additional samples from revertant tissue, we established a greenhouse plot of plants expressing the *glo-1* unstable allele for several years, and obtained

revertant flowers with second-whorl chimeric organs (Figure 3h–j) during the spring under southern Spain growing conditions.

We analyzed the levels of *DEF* and *GLO* expression in the second-whorl organs of the transgenic line displaying the strongest phenotype by quantitative RT-qPCR, and found that expression was reduced to 2% of wild-type, as expected for organs that were completely transformed into sepals (Figure 5). The smallest second-whorl chimeric organs recovered (sepal/petal) displayed levels of *DEF* expression at a level that was 14% of that of the wild-type (P = 0.029) (Figure 5a). Furthermore, the two independent RNAi lines displayed an interesting feature in that the first flowers showed a *def* phenotype that was lost later on, indicating that an acropetal gradient could overcome the RNAi-dependent decrease in gene expression. Independent transgenic experiments with other genes indicate that this was a feature of the *RNAi-DEF* construct but is not a general feature of *Antirrhinum* stably transformed lines (Manchado-Rojo, M., unpublished observations). *RNAi-DEF* flowers with a wild-type appearance had *DEF* and *GLO* expression levels similar to wild-type. The *glo-1* allele shows instability, and we compared it against the *RNAi-DEF* plants and the series of *co* and def^{nic} mutants. The results obtained show that the levels of *DEF* in *glo-1* were between 11 and 31% of wild-type in sepal and sepal/petal organs, with 22% in revertant petals and wild-type levels of expression in near-wild-type looking petals. In contrast, *GLO* levels did not fully recover the wild-type expression levels, showing levels that were always significantly lower than in wild-type petals.

Our data shows that thresholds of 11–15% of wild-type levels of expression of *DEF* or *GLO* are associated with development of recognizable petal tissue.

Reciprocal *DEF* and *GLO* transcript levels change during development

Although the currently supported hypothesis of B function is based on a positive autoregulatory loop of two B function gene products, a direct comparison of mRNA levels for *DEF* versus *GLO* has not been reported, and examination of the levels of *DEF* and *GLO* in the tissues analyzed indicated that important differences may exist in the reciprocal levels of expression. Thus we used the data obtained to perform a nested calculation allowing direct comparison of *DEF* versus *GLO* in each sample. We used a large sample for quantitative RT-qPCR: 20 biological samples with three technical replicates comprising ten wild-type flowers at developmental stage 13–14, i.e. approximately 1 cm long and still closed (Vincent and Coen, 2004), and ten wild-type fully open flowers 1 day after anthesis. The reason for this was that sampling of revertant and transgenic tissue necessarily has to be performed when development is complete and the phenotypes are distinguishable, but we wished to identify possible ontogenic changes in reciprocal levels of *DEF* and *GLO* gene expression. A

simple inspection of the data from closed flowers showed that, contrary to what was expected, transcriptional levels of *DEF* and *GLO* were not equal. *GLO* gene expression in petals was significantly lower than that of *DEF* in four of ten samples. Combining all samples indicated a level for *GLO* transcripts of 0.806 relative to *DEF* (P = 0.013), with most values below 1.0 (Figure 6 and Table 6). Surprisingly this unequal relationship for *GLO* versus *DEF* expression levels resulted in significantly higher expression of *GLO* in petals when flowers were open (1.847; P = 0.000). We compared two samples of gene expression data that had equal variances (Fligner–Killeen test, P = 0.5541), and found that, as expected from the data inspection, the relationships between *DEF* and *GLO* expression in closed and open flowers are significantly different (*t*-test, P = 5.765e-06). The data show that, from middle to late stages of development, the relationship between *DEF* and *GLO* transcription varies significantly, with a marked up-regulation of *GLO* compared to *DEF*.

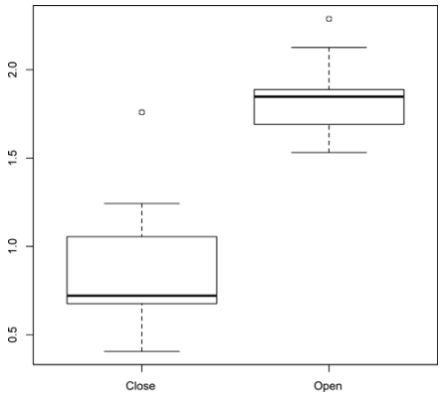

Figure 6. Box plot of expression values for *GLO* versus *DEF* in closed and open flowers. The *y* axis refers to *GLO* expression values compared to *DEF* having an arbitrary value of 1.

Table 6. Expression of *GLO* compared to *DEF* in various tissues

Organ	Related *GLO* expression	p values
WT close	0.806	0.013
WT open	1.847	0.000
CO	13.375	0.001
DEF^{nic}	20.128	0.0020
CO DEF^{nic}	6.824	0.0010
pH12-DEF petal (small)	6.465	0.003
pH12-DEF petal-sepal	98.632	0.001
pH12-DEF sepal	60.537	0.0000
pH12-DEF tube	8.05	0.234
pH12-DEF (normal flower)	12.36	0.0010
Glo-1 sepal	0.093	0.001
Glo-1 rev	0.617	0.491
Glo-1 petal	1.057	0.695

An arbitrary value of 1 was assigned to each tissue for the level of *DEF*.

In *glo-1* revertant petals, *DEF* and *GLO* expression levels were close to 1. However, *DEF* and *GLO* levels were dissimilar in the rest of the samples analyzed (Table 6). In perfectly formed petals of

RNAi-DEF plants, *GLO* expression was at least 11-fold higher than *DEF*. These large differences between *DEF* and *GLO* expression were also found in *co* petals, indicating that disparity in gene expression between *DEF* and *GLO* may be tolerated and still give rise to petal tissue. In the rest of the samples with strong homeotic alterations, differences between *DEF* and *GLO* ranged between five fold in *co def*^{nic} double mutants to close to 100-fold in the strongest homeotically transformed organs, i.e. sepal/petal organs of *RNAi-DEF* or second-whorl sepals of *glo-1*. Our results show that, in wild-type flowers, *DEF* and *GLO* expression is not matched, and the large differences between the two genes in terms of gene expression in the array of tissues analyzed cannot be completely reconciled if we assume a positive autoregulatory loop as the sole form of B function transcriptional maintenance.

Effect of the *co* mutation and B function manipulation on downstream processes

As *MIXTA* is a well-defined downstream target of B function, and the *co* mutation affected the area of conical cells, we measured *MIXTA* gene expression and found that levels of *MIXTA* in the *co* mutant were as low as 4.1% of the wild-type (Figure 5). These low levels were also found in second-whorl sepals of *glo-1*, *glo-1* revertant sepal/petal tissues and *RNAi-DEF* normal flowers. However, the other *RNAi-DEF* tissues showed higher levels of *MIXTA* expression, indicating that, although the levels of *DEF* and *GLO* were significantly lower than in wild-type, there may be other factors involved requiring further analysis.

Discussion

A quantitative component of homeotic gene function

As the ABC model is based on spatial restriction of gene expression, much information has been generated to explain the discrete gene expression patterns. Many mutants identified show homeotic changes caused by lack of expression of the ABC genes. Less well characterized are the quantitative requirements for floral organ identity genes. The original hypothesis developed in *Antirrhinum* postulates that *DEF* and *GLO* transcription occurs in an initial step, and self-maintained gene expression levels take over the initial activation to run the developmental program until organ development is complete.

Our data shows that levels of *DEF* or *GLO* mRNA of 11% or above can support development of recognizable petal tissue. However, these levels do not sustain full organ size. The fact that the palate is completely absent in many *def*^{nic} flowers and all *co def*^{nic} double mutants indicates that different regions of the petal also require different thresholds of B function for development or have

different levels of expression of B function along the petal area. The effects on late developmental stages are even more pronounced, as the finding of *MIXTA* expression levels of 4% of the wild-type in the *co* mutant or 16% in *RNAi-DEF* revertant petal confirms previous work that established the quantitative importance of *DEF* expression for *MIXTA* expression (Perez-Rodriguez *et al.*, 2005). Reduced expression of the C function gene *AGAMOUS* (*AG*) in *Arabidopsis* by RNAi plants and plants expressing several *ag* alleles analyzed have shown that threshold levels of *AG* have different effects on organ identity and meristem determinacy (Sieburth *et al.*, 1995;Causier *et al.*, 2009; Das *et al.*, 2009; Maier *et al.*, 2009), indicating that not all downstream processes require the same levels of expression. Furthermore, quantitative changes in gene expression modify the spatial expression of *AG* in *Arabidopsis* (Cartolano *et al.*, 2009), supporting the importance of quantitative gene expression levels for floral patterning and organ development.

The phenotypic effects of the *co* mutation on petal cell development clearly show a strong decrease in the area with conical cells, correlated with down-regulation of *MIXTA*. This decrease in the area comprising conical cells and the smaller size of the cells may explain the decrease in *MIXTA* expression. It may also explain a quantitative decrease in scent production, as benzoic acid carboxyl methyl transferase, which is involved in methyl benzoate production, is expressed in conical cells in *Antirrhinum* (Kolosova *et al.*, 2001). An additional role of *CO* downstream of *DEF* is supported by the finding that def^{nic} single mutants and *co* def^{nic} double mutants have similar levels of *DEF* and *GLO*, but the phenotypes analyzed are more extreme in the double mutant, suggesting that *CO* is a B function gene that is involved in activation or maintenance of *DEF*, and activation of part of the scent transcriptional network at late stages of petal development.

Determining the degree of homeotic transformation or petal organ identity has not been an easy task. Studies in *Arabidopsis* using ectopic expression of *PISTILLATA* (*PI*) or its homolog from pea *PsPI* have used chimeric first-whorl organs comprising sepal and petal tissue as criteria to establish B function activity (Krizek and Meyerowitz, 1996; Berbel *et al.*, 2005). Indeed, model organisms such as *Petunia*, in which B function genes are duplicated, allow a much more detailed analysis. For instance, petal defects in plants expressing mutant alleles of *Phglo1* and *Phglo2* show greener and broader midvein, conversion of conical cells to sepal-like epidermal cells, or lack of stamen fusion to the petal tube (Vandenbussche *et al.*, 2004). Expression of the *TM6* gene from *Petunia* under the control of a 35S promoter can rescue *Phdef* phenotypic defects to some extent, but still petals show a broad green midvein (Rijpkema *et al.*, 2006). Thus our approach of considering petal tissue as second-whorl organs that have recognizable regions resembling petals and are confirmed by scanning electron microscopy as having conical cells, fulfils a qualitative definition of the organ.

Determining what a petal is in terms of identity does not address the functional aspects. Petals have at least two recognizable functions: physical protection of the sexual organs during flower development, and insect attraction. Clearly these two functions do not necessarily overlap or have similar importance in all plants. Our data suggest that levels of *DEF* and *GLO* transcription above a threshold are required to obtain wild-type petal size, good development of conical cells, and scent production. Our data do not allow us to determine whether the reduction in scent is a result of decreased *MIXTA* expression that leads to fewer conical cells in which scent is produced, or whether it is a direct effect of *CO* or *DEF* and *GLO*. However, these possibilities are not mutually exclusive.

Structure of the B function regulatory network

A network topology based on a simple positive autoregulatory loop is not supported by the data obtained in the various tissues and genetic backgrounds described here. First, the *co* mutant has a strong effect on *DEF* but not on *GLO* gene expression, indicating that *CO* may be directly involved in *DEF* activation. *GLO* expression levels were maintained despite a decreased level of *DEF–GLO* heterodimer and increased basal promoter activity, indicating that other factors maintain *GLO* expression in the absence of positive autoregulation. This basal promoter activity may be flower-specific as *GLO* has well-defined expression patterns in petals and stamens (Trobner *et al.*, 1992; Zachgo *et al.*, 1995). In second-whorl organs with complete homeotic transformation, although the actual level of expression of *DEF* in *glo-1* or *GLO* in *RNAi-DEF* is significantly down-regulated compared to wild-type, the levels of expression of *GLO* compared to *DEF* in *RNAi-DEF* and *DEF* versus *GLO* in *glo-1* are significantly higher. This implies that the positive autoregulatory loop is only part of the B function maintenance, and a basal level of transcriptional activation is present for both *DEF* and *GLO*, at least at late stages of development. A graphical model describing the current model of transcriptional network and a new one based on the data presented in this contribution are shown in Figure 7. Activation of B function consists of triggering *DEF* and *GLO* (Figure 7a), and such activation becomes independent at the middle and late stages of development (Figure 7b). Activation of *DEF* and *GLO* seem to be partly independent as *CO* only affects *DEF* (Figure 7c). Early experiments in tobacco showed a spatial difference in transcriptional activation of *NtDEF* and *NtGLO.* Ectopic expression of *DEF* and *GLO* causes ectopic expression of *NtDEF* in leaves and all floral organs, whereas *NtGLO* is ectopically expressed only in first-whorl organs (Davies *et al.*, 1996a). This indicates that positive autoregulation comprises organ-specific components and may differ between *DEF* and *GLO.*

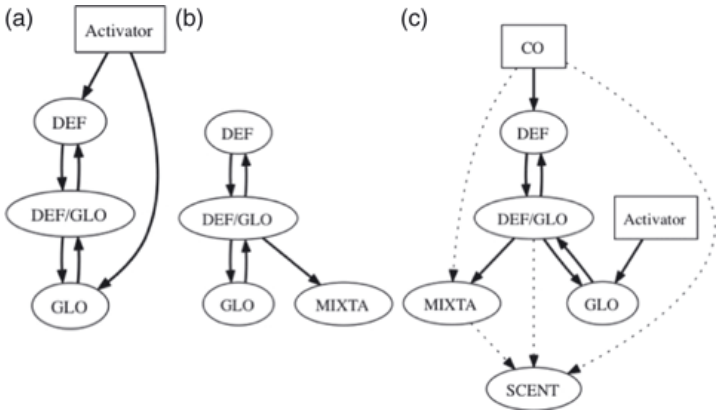

Figure 7. A graphical model of B function network topologies. Initial steps of B function activation during early petal development (a), middle to late stages of petal development (b), and a newly proposed network topology for late stages of development (c). Rectangles refer to a transcriptional activation function. Dotted lines refer to direct activation that is possible but the current data do not allow discrimination between direct and indirect activation.

We did not expect to observe differences in gene expression between *DEF* and *GLO*, and even more unexpected was the existence of developmental differences such as those found in the balance of the two transcripts. This also shows that the transcriptional network topology of B function changes during petal development, and is not a fixed entity as originally thought. However, petal growth and anthocyanin production are biphasic. Petal growth comprises cell division in early stages and cell expansion at later stages in different plants (Martin and Gerats, 1993; Reale *et al.*, 2002), whereas anthocyanin genes show early and late activation (Jackson *et al.*, 1992;Weiss *et al.*, 1993). Finally, scent production starts at anthesis. These obvious changes in the downstream targets of *DEF* and *GLO* cannot be explained by a simple model in which B function operates as a single-geared process. Our work has concentrated on transcriptional changes, but recent work has shown that translation rate constants play a dominant role in determining protein levels, and, combined with mRNA levels, account for 95% of the variance in protein quantities (Schwanhäusser *et al.*, 2011). This suggests that, if linear levels of translation rates are maintained during petal development for *DEF* and *GLO*, the changes in mRNA should translate into differing levels of protein.

Recent work has shown that quaternary complexes with diverse composition co-exist in *Arabidopsis* petals, indicating that there is an inherent flexibility in MADS-box tetramer formation (Smaczniak *et al.*, 2012). The developmental changes in the ratio of *DEF*/*GLO* expression levels may have implications with regard to the type of target genes that plant MADS box complexes recognize during petal development, and may explain the gradual developmental processes that occur during petal morphogenesis that lasts more than 20 days in *Antirrhinum*.

Experimental Procedures

Plant material and genetics

The *compacta* and *deficiens nicotianoides* mutants were obtained from the Gatersleben collection (IPK Gatersleben, Gatersleben, Germany). The laboratory lines Sippe50, 165E and the *globosa* unstable allele *glo-1* (Trobner *et al.*, 1992) was obtained from Dr Zsuzsanna Schwarz-Sommer (Max-Planck-Institut für Pflanzenzüchtungsforschung, Köln, Germany). Plants were grown in the greenhouse as described previously (Bayo-Canha *et al.*, 2007). Homozygote mutants were crossed to obtain F_2 plants as described previously (Egea-Cortines *et al.*, 1999), and double mutants were identified by their phenotype (see Results) and the corresponding Mendelian segregation.

Microscopy

Fully developed flowers were harvested and analyzed as described previously (Delgado-Benarroch *et al.*, 2009b).

Constructs

We cloned a fragment of 207 bp encompassing the last 60 codons and 27 bp of the 3' UTR of the *DEFICIENS* cDNA from *A. majus* into the pHellsgate12 plasmid (Helliwell and Waterhouse, 2003), using the primers *DEF*-forward (5'-GATGCAAGGAGAGAGGATC-3') and *DEF*-reverse (5'-CTATAACATATATCGATCATACCATTAATT-3') (Table S1). The hairpin construct of the pHellsgate12 vector was checked by PCR using an internal primer for the intron pH12-forward (5'-GTTGGCAGCATCACCCGA-3') and pH12-reverse (5'-AAACTAGAAATTTACCTGCAC-3') and a primer for the *DEF* gene in both directions.

Scent analysis

The volatile constituents in the flowers of the plants were separated and qualitatively identified by capillary gas chromatography/mass spectrometry (GC-MS).

For extraction of the volatile components, one cut flower per line was placed inside Falcon tubes for 24 h (DeltaLab,http://www.deltalab.es). The tubes contained a suspended Twister™ bar (Gerstel GmbH & Co. KG, http://www.gerstel.de/), a magnetic stir bar of 10 mm length coated with 0.5 mm polydimethylsiloxane that had previously been conditioned.

Scent profiles were resolved on a 6890 gas chromatograph coupled to a 5975 inert XL mass selective detector (Agilent Technologies,http://www.home.agilent.com) equipped with a thermal desorption unit, a cooled injector system (CIS 4) and a multi-purpose sampler (MPS2) (Gerstel GmbH & Co. KG).

The GC separation was performed on an HP-5MS UI capillary column (Agilent Technologies), 30 m, length × 0.25 mm, internal diameter x 0.25 µm (film) in constant pressure mode. The oven temperature was sequentially increased from 50 to 70°C at 5º per min, held for 1 min, and thereafter increased to 240°C at 10°C per min, with a holding time of 15 min. The inlet operated in solvent vent mode with a split ratio of 1:15. Chromatographic-grade helium was used as the carrier gas. We used *n*-pentadecane as an internal standard for qualitative analysis of the samples, adding 1 µl *n*-pentadecane (standard for gas chromatography, Fluka, Sigma-Aldrich,http://www.sigmaaldrich.com) prepared to 20 ppm in dichloromethane (Lab-Scan, http://www.labscan.ie/)).

The stir bar was thermally desorbed into the thermal desorption unit using the following desorption temperature program: initial temperature of 40°C, ramping at 100°C per min until 150°C, and a holding time of 5 min. The transfer temperature was 300°C, working in splitless desorption mode. The volatiles thermally desorbed were cryo-focused in the cooled injector system inlet at −100°C using liquid nitrogen, with a carrier gas flow of 50 ml min^{-1}. After cryo-focusing was completed, the volatiles were transferred into the capillary column by heating the CIS4 inlet at a rate of 10°C sec^{-1} to 150°C (holding time 3 min).

Mass spectra were collected in the scan range *m*/*z* 30–450. The measurements were performed using an electron bombardment ion source with electron energy of 70 eV. The transfer line, source and quadrupole temperatures were set at 280, 230 and 150°C, respectively. The chromatograms and mass spectra were evaluated using ChemStation software (G1791CA, version D.03.00; Agilent Technologies). Chromatographic peak identification was performed by library matching using the Standard Reference Database 1A NIST 2005, version 2.0 (National Institute of Standards and Technology, http://www.nist.gov/rsd/nist1a.cfm).

Quantitative PCR

Total RNA was isolated from 100 mg homogenized plant material using an RNeasy mini kit (Qiagen, http://www.qiagen.com/default.aspx), including DNase treatment. cDNA was synthesized from 1 µg total RNA using a Maxima® first strand cDNA synthesis kit (Fermentas, http://www.fermentas.com/en/home).

Quantitative RT-PCR reactions were performed using SYBR Premix ExTaq™ (Takara, http://www.takara-bio.com/) on a Rotor-Gene Q machine (Qiagen). The housekeeping gene *ubiquitin protein ligase* was used for relative quantification of gene expression. In order to minimize the variability, we used three biological replicates and two technical replicates for each sample. We obtained take-offs and efficiency values and computed differences in gene expression analysis as described previously and using the REST program (Pfaffl*et al.*, 2002; Delgado-Benarroch *et al.*, 2009a; Mallona *et al.*, 2010, 2011).

Antirrhinum transformation

We developed a new protocol to obtain stable transformants (Appendix S1). These transformants were further analyzed by PCR using primers for the *NPTII* gene. Two independent plants positive for *NPTII* that showed phenotypes that ranged from the classic null allele def^{gli} to weak alleles such as def^{nic} were also used.

Statistics

Statistical analysis was performed using the R package (http://www.r-project.org/) and Excel (Microsoft, http://www.microsoft.com). Unless otherwise stated, we used the Kruskal–Wallis test because growth and cellular data were not normally distributed.

Graphical modelling

The graphic models describing the currently known and proposed transcriptional networks were programmed in the Dot graph specification language and visualized using Graphviz (http://www.graphviz.org).

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Supporting Information

Figure S1. Segregation of colour genes in a *compacta* genetic background. The genes *delila*, *nivea* and *pallida* recurrens segregate in a cross of the *Antirrhinum majus* lines Sippe50 x 165E. Flowers of *co* on the left and wild-type on the right with wild-type color (a); double mutant *niv pal* (b); and *delila* (c).

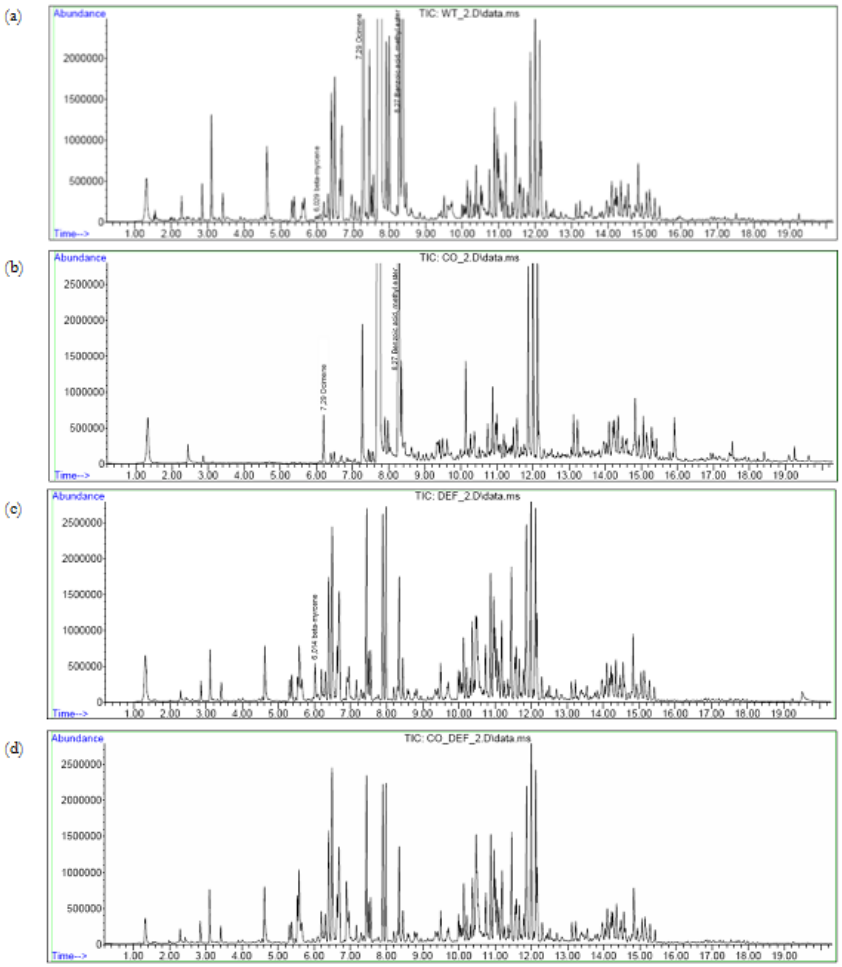

Figure S2. Volatile profiles of wild-type*, co, def*^{nic} *and co def*^{nic} *double mutants*. Chromatographic peak identification of the *Antirrhinum majus* scent profiles. The peak resolved at retention times of 6.029 for myrcene, 7.29 for ocimene and 8.27 for methyl benzoate. Chromatograms correspond to (a) Wild-type; (b) *co*; (c) *def* and (d) *co def*^{nic} double mutants.

Figure S3. Additional *RNAi-DEF* line with a weak phenotype.

Table S1. Primers for RT-qPCR and cloning.

PRIMER	SEQUENCE
Am DEF forward	5' GATGCAAGGAGAGAGGATC 3'
Am DEF reverse	5' CTATAACATATATCGATCATACCATTAATT 3'
Am GLO forward	5' TTGTCCGGATGATGAGG 3'
Am GLO reverse	5' CGGAACGCGAAAGG 3'
Am MIXTA forward	5' CACCAACTACTCCGCACGTCC 3'
Am MIXTA reverse	5' CCATTGACGACGACGAGGCC 3'
Am Ubiq. forward	5' GCCGATGGAAGTATATGTTTGGACATC 3'
Am Ubiq. reverse	5' CTAACTTTGCGGTTATAATCTCGTTTA 3'
pH12 forward	5'-GTTGGCAGCATCACCCGA-3'
pH12 reverse	5'-AAACTAGAAATTTACCTGCAC-3'

Appendix S1. Detailed *Antirrhinum majus* transformation protocol.

Development of a reliable transformation protocol for Antirrhinum

One major problem in the development of *Antirrhinum* as a model in the last decade has been the difficulty of developing a reliable transformation protocol for transgenic approaches (Heidmann *et al.* 1998) Previous studies in *Antirrhinum* have used hypocotyls as explants for transformation (Cui *et al.* 2004). Growth of hypocotyls, is partly due to cell expansion and it has been shown that in the dark, there is considerable endoreduplication in *Arabidopsis* hypocotyls (Gendreau *et al.* 1997). This suggests that using old hypocotyls might encounter the problem of obtaining explants with a mesoploid structure, where the number of diploid cells able to regenerate might decrease with time. We tested the developmental window of *Antirrhinum majus* hypocotyls as explants for regeneration and transformation. After about two weeks, hypocotyls that belonged to the four-week old treatment had not shown any sign of regeneration whereas regeneration occurred in the plates with hypocotyls from two-week old germinated seedlings. After about 5 weeks, the difference between two and four-week old hypocotyls, was seen by visual inspection. Whilst most hypocotyls from two-week old explants had produced calluses four-week old explants were not reactive to the regeneration medium and the percentage producing calluses was close to zero.

Antirrhinum majus transformation

PLANT MATERIAL AND AGROBACTERIUM STRAIN

We have used the laboratory line 165E for transformation. The *Agrobacterium* strain used is EHA105.

SEED STERILIZATION AND IN VITRO CULTIVATION:

- Place the seeds in a microfuge tube.
- Add 1 ml 70% ethanol to the tube and vortex.
- Remove ethanol with a 200 µl pipette (seeds are larger than the tip).
- Add 1 ml 20% bleach + detergent. Occasionally mix the seeds. After 10 min remove the bleach with a 200 µl pipette.
- Rinse the seeds with sterile distilled water, three times for 5 minutes. Remove the water using a 200 µl pipette each time.
- Put the seeds into a Petri dish with MS-medium.
- Grow 2 weeks.

MEDIUM:

- SIM medium (per litre):
    - Sodium citrate $2H_2O$ 5.882 g
    - Sucrose 20.0 g
    - Acetosyringone 500 µM
    - Adjust the pH to 5.5
    - Sterilize by autoclaving for 15 minutes at 121ºC
- Murashige & Skoog medium (per litre):
    - MS 4.4 g
    - Sucrose 25.0 g
    - Gelrite 2.5 g
    - Adjust the pH to 5.7 – 5.9
    - Sterilize by autoclaving for 15 minutes at 121ºC
- Co-cultivation medium (per litre):
    - MS 4.4 g
    - Sucrose 25.0 g
    - Gelrite 2.5 g
    - Adjust the pH to 5.7 – 5.9
    - Sterilize by autoclaving for 15 minutes at 121ºC. After letting it cool down to 50ºC aprox. add:
        - NAA 0.25 mg
        - Zeatine 2.0 mg
        - Acetosyringone 100 µM
- Tras-cultivation medium (per litre):
    - MS 4.4 g
    - Sucrose 25.0 g
    - Gelrite 2.5 g
    - Adjust the pH to 5.7 – 5.9
    - Sterilize by autoclaving for 15 minutes at 121ºC. After letting it cool down to 50ºC aprox. add:
        - NAA 0.25 mg
        - Zeatine 2.0 mg
        - Cefotaxime 300 mg
        - Kanamycin 100 mg

  - Vancomycin 200 mg

- Root medium (per litre):

  - MS 4.4 g
  - Sucrose 25.0 g
  - Gelrite 2.5 g
  - Adjust the pH to 5.7 – 5.9
  - Sterilize by autoclaving for 15 minutes at 121ºC. After letting it cool down to 50ºC aprox. add:
    - NAA 0.20 mg
    - Cefotaxime 300 mg
    - Kanamycin 50 mg
    - Vancomycin 200 mg

MATERIALS

- Co-cultive medium liquid
- Sterile circles of filter paper as big as a Petri plate
- Scalpels and forcepses

PREPARATION

DAY 0

- Friday before, spread the Agrobacterium containing your plasmid in a Petri plate with fresh medium and the appropriate antibiotics. Grow it for the weekend at 27ºC.

DAY 3

- Monday, put a colony in 10 ml LB with antibiotics at midday. Grow it o.n. at 27ºC and shaking at 180 rpm.

DAY 4

- Tuesday, centrifuge the Agrobacterium culture at 3000 rpm for 15 min in a Falcon.
- Remove the supernatant quickly and carefully.
- Resuspend the pellet in 10 ml SIM medium without antibiotics or AS
- Measure the OD_{600nm}, it should be in stationary phase.
- Dilute the culture to OD_{600nm} = 0.2 in a final volume of 25 ml of SIM medium with antibiotics and AS 500 µM
- Grow the culture for 24 hours at 25ºC shaking at 180 rpm.

DAY 5

- Centrifugate the culture at 3000 rpm for 15 min in a Falcon
- Remove the supernatant quickly and carefully.
- Resuspend the pellet in 25 ml liquid co-cultive medium (without gelrite), without antibiotics.
- Measure the OD_{600nm}
- Dilute the culture in liquid co-cultive medium until OD_{600nm}= 0.02.

TRANSFORMATION

- Excise the hypocotyls/leaves from seedling and place on a filter paper lined co-cultivation medium plate.
- Add 10 ml of Agrobacterium diluted in liquid co-cultive medium until OD_{600nm}= 0.02 to each Petri plate and leave it for 15 min.
- Remove the Agrobacterium solution with a sterile disposable syringe. Dry the explants with sterile filter paper.
- Seal plate with parafilm and incubate in the dark for 2 days in the growth chamber.

DAY 7

- Prepare fresh trans-culture medium plates.
- Transfer the explants to these selective medium plates (25 per plate). Seal with parafilm. Incubate in the dark for a week, and then start to adapt them to full light by putting on growth chamber with light. Pile on the top 4-5 sheets of filter paper and remove one per day till full light is achieved.

DAY 20

- Transfer the explants to fresh trans-culture medium plates.

2 MONTHS…

- After 6-8 weeks shoots should appear. Transfer the explants each 20 days to fresh selection plates with trans-culture fresh medium. Excise shoots of 1.5 – 2.0 cm, and put them on root medium.

Major pitfalls encountered include:

1- Old seed failing to germinate or excess of bleach treatment causing lack of germination.
2- Transgenic plant death after regeneration and during the adaptation to the greenhouse.

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Chapter 3

Chapter 3: Validation of *Aintegumenta* as a gene to modify floral size in ornamental plants

María Manchado-Rojo[1]; Julia Weiss[1] and Marcos Egea-Cortines[1]*

Genetics, ETSIA, Instituto de Biotecnología Vegetal, Universidad Politécnica de Cartagena, Paseo Alfonso XIII 48, 30203 Cartagena, Spain.

*Correspondence: (Telf.: +34868071077; FAX +34968325433; e-mail: marcos.egea@upct.es)

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Summary

The gene *AINTEGUMENTA* (*AtANT*) is an *APETALA2* transcription factor in *Arabidopsis* activating growth downstream of auxin signalling. Lateral organ size is positively correlated with *ANT* expression in *Arabidopsis*. We tested the use of *AtANT* as a tool to modify floral size in two different plants used as model organisms and ornamental crops, *Petuniaxhybrida* and *Antirrhinum majus*. *Petunia* plants expressing *PhANT* RNAi showed a decrease in *PhANT* expression correlated with smaller petal limbs. In contrast *Petunia* plants overexpressing *AtANT* had larger petal limbs. Petal tube length was less affected in downregulation of *PhANT* or overexpression of *AtANT*. Overexpression of *AtANT* in *Antirrhinum* caused increased flower size via increased petal limb width and tube length. Downregulation of *PhANT* showed an effect on cell size while overexpression of *AtANT* in *Petunia* and *Antirrhinum* caused significant increases in cell expansion that could explain the differences in floral organ size. The endogenous expression levels of *PhANT* and *AmANT* tended to be higher in the limb than in the tube in both *Antirrhinum* and *Petunia*. *AtANT* overexpression caused significant *AmANT* upregulation in *Antirrhinum* limbs but not of *PhANT* in *Petunia*, indicating differences in the regulatory network. The differential effect of *AtANT* on limb and tube in *Petunia* and *Antirrhinum* correspond to phenotypic differences observed in natural variation in the corresponding genus indicating a relation between the phenotypic space of a genus and the effect of modified *ANT* levels, validating *ANT* as a gene to modify floral size.

Introduction

Floral size is a trait subject to strong selection in those species that display an allogamous reproductive strategy coupled to insect-based pollination (Krizek and Anderson, 2013). The genus *Petunia* for instance shows a distinct pollination syndrome with a suite of traits that attract hummingbirds or hawk moths including floral colour, scent and size (Hermann and Kuhlemeier, 2011). Changes in floral size can be genetically dissected into two separate regions, the tube and the limb that seem to play distinct roles in pollination (Stuurman *et al.*, 2004; Venail *et al.*, 2010). In the genus *Antirrhinum*, there is an important range in flower size indicating local adaptation to insect pollinators (Feng *et al.*, 2009). *Petunia* and *Antirrhinum* are used as model systems but are also sold as ornamentals. As a result they have undergone major screens for traits including amongst others increased and decreased floral size, depending on the market requirements.

A proper flower shape and size requires correct floral organ identity as mutations in genes affecting organ identity strongly affect cell division and expansion (Egea-Cortines and Weiss, 2013; Dornelas *et al.*, 2011; Delgado-Benarroch *et al.*, 2010; Manchado-Rojo *et al.*, 2012). A model for floral

development was originally proposed with the so-called A, B and C combinatorial functions (Coen and Meyerowitz, 1991).The A function is fulfilled in *Arabidopsis* by the *APETALA1* and *APETALA2* genes, but the level of implication of the corresponding orthologs in petal development varies to a large extent in different plants including *Petunia* and *Antirrhinum* (Maes *et al.*, 2001; Keck *et al.*, 2003; Egea Gutierrez-Cortines *et al.*, 2000; Causier *et al.*, 2010). Petal development occurs mainly by activation of the so-called B-function organ identity genes that form ternary complexes with other MADS box genes (Causier *et al.*, 2003; Egea-Cortines *et al.*, 1999; Ferrario *et al.*, 2003; Honma and Goto, 2001). Several studies have shown that petal development occurs by an early phase of cell division followed by a late stage of cell expansion (Reale *et al.*, 2002). Thus modified floral size can be the result of increased cell proliferation and/or cell expansion. Current data in *Arabidopsis* and *Antirrhinum* indicate that modified periods of cell proliferation produce overall changes in floral size (Delgado-Benarroch*et al.*, 2009a; Disch *et al.*, 2006; Szecsi *et al.*, 2006). Genes involved in control of cell expansion also cause changes in petal size. For example, regulatory genes like *SUPERMAN (SUP)* have been shown to repress cell expansion in *Petunia* and *Arabidopsis* flowers (Kater *et al.*, 2000), indicating a tight control of cell size during flower development. Genes directly controlling cell wall metabolism also show dose response effects in petal size as in case of *Petunia*, where down regulation of the *EXPANSIN* (*EXP*)*. PhEXP* causes a strong decrease in petal size as a result of reduced cell size (Zenoni *et al.*, 2004). In contrast, overexpression of the same gene causes increased petal limb size via enhanced cell enlargement (Zenoni *et al.*, 2011).

Floral size changes can be achieved by increasing the number of organs, the size of the organs or both parameters. Changes in the number of petals as a result of changes in organ identity and loss of floral meristem determinacy are typical of loss of C-function in *Arabidopsis* and *Antirrhinum* (Bradley *et al.*, 1993; Yanofsky *et al.*, 1990). Changes in petal number leading to double flowers is correlated in the ranunculid *Thalictrum thalictroides* with loss of protein-protein interaction between a C-function *AGAMOUS* gene (*ThtAG1*) and its *SEPALLATA* partner (*ThtSEP3*) (Galimba *et al.*, 2012). Further evidence for changes in C-function has been described in rose where allele-specific restriction of the expression domain of the C-function *AGAMOUS* (*RhAG*) is correlated with increases in the number of petal whorls (Dubois *et al.*, 2010). A second group of ornamentals show changes as a result of modifying floral organ size. This is typically seen in flowers like orchids, *Petunia* or *Antirrhinum* (Gawenda *et al.*, 2012; Weiss *et al.*, 2005). The genes involved in changes in floral organ size in ornamentals have not been described.

The gene *AINTEGUMENTA* (*ANT*) is an *APETALA2* transcription factor, originally identified in a screen for female sterile plants, and is required for ovule initiation in *Arabidopsis* (Klucher *et al.*, 1996; Elliott

et al., 1996). Later work has shown that the overexpression of *ANT* in *Arabidopsis* causes increases in organ size (Krizek, 1999; Mizukami and Fischer, 2000). The *ANT* group comprises the *AINTEGUMENTA-LIKE* (*AIL*) genes involved in growth-related processes in a variety of plants. Expression of *AIL* genes correlate with nucellar development in coconut (Dona *et al.*, 2010). Overexpression of *AIL-6* in *Arabidopsis* shows a variety of phenotypes including modified floral size (Krizek and Eaddy, 2011), indicating a general role of *ANT* and *AIL* genes in growth control. The *PLETHORA* (*PLT*) genes form a second group of the *AP2* transcription factors involved in root stem cell maintenance and shoot phyllotaxis (Galinha *et al.*, 2007; Prasad *et al.*, 2011; Aida *et al.*, 2004). A gene duplication event in *Arabidopsis* points towards a role of *ANT* in overall growth control in a dose-response fashion (Horiguchi *et al.*, 2009). Experiments with the mutant *argos (arg)* (Hu *et al.*, 2003) places *ANT* downstream of *ARG* transducing auxin signals related to growth promotion. Expression of *ANT* is thought to control meristematic competence (Mizukami and Fischer, 2000), thus affecting the timing of cell division. Although the function of *ANT* makes it an attractive candidate gene to modify lateral organ growth in plants, there is no data available outside *Arabidopsis*.

Here we show that modifying the expression levels of *ANT* in two ornamental plants, *Petunia* and *Antirrhinum*, causes changes in floral size. We down-regulated *PhANT* in *Petunia* using a *PhANT* RNAi construct leading to decreased floral size. We overexpressed the *Arabidopsis AtANT* gene in *Petunia* and *Antirrhinum*. *Petunia* plants displayed increased petal limb and small increases in petal tube whereas *Antirrhinum* showed increased petal tube and petal limb width resembling the natural phenotypic variability that exists within each genus. Floral size phenotypes resulting from *AtANT* overexpression were caused mainly by increased cell expansion. The effect of *AtANT* on the endogenous levels of *PhANT* and *AmANT* also differed with a significant up-regulation of *AmANT* but not *PhANT,* indicating differences in the *ANT* regulation of tube and limb and between *Petunia* and *Antirrhinum*. We discuss our results in terms of the genus specific genetic context, and the biotechnological potential of *ANT* as a gene to modify floral size.

Results

Cloning of an *AINTEGUMENTA* gene fragment from *Petunia* for functional analysis

We defined two experiments to evaluate *ANT* as a gene to manipulate floral size in ornamentals. One based on loss of function via a RNAi construct and by gain of function of the *Arabidopsis ANT* gene. We needed first to isolate the *Petunia ANT* (*PhANT*) to develop RNAi constructs. In order to identify an *ANT* clone from *Petunia* we searched the Solanaceae public database (solgenomics.net) using the *ANT* gene from *Arabidopsis.* We were not able to identify a homolog of *Petuniaxhybrida* but we

retrieved the sequence of a tomato clone (SGN-U599088 Tomato 200607) with high homology (E Value 3e-89; Identity 85%) to the *ANT* gene from *Arabidopsis*. Previous work in our laboratory had shown that direct usage of primer combinations from tomato in *Petunia* had over 60% of success rate (unpublished data). Thus we amplified and cloned a *Petunia* fragment using primers designed on the basis of tomato sequence. Sequence comparison showed that the *Petunia* fragment was virtually identical to the tomato sequence from the database. We performed standard RACE PCR and obtained several fragments that together comprise 1.2 kb of coding sequence of *PhANT* (Genebank accession AHC98702).The isolated fragment comprising identified as closest homolog the *ANT* gene from *Arabidopsis* in the *Arabidopsis* genome (BLASTP 1e-97). A direct comparison of the *PhANT* translated protein showed a degree of identity of 38.9 % with *AtANT* and of 27.3 % with *AIL*-5.The *ANT* gene belongs to the *AP2* family of transcription factors, divided in the *euAP2* and *ANT* lineages (Kim *et al.*, 2006; Shigyo *et al.*, 2006; Shigyo and Ito, 2004). In order to confirm the homology of the isolated clone to the *ANT* lineage, we performed a phylogenetic analysis using predicted proteins from the *AP2ANT* family. These included genes of the *AP2* lineage, *PLETHORA (PLT)* and *CBF*/*DREB* involved in cold response (Jaglo-Ottosen *et al.*, 1998) (Table S1). We performed a phylogenetic analysis using two open source phylogenetic software pipelines with identical results (see materials and methods). The phylogenetic tree obtained comprised four major clades (Figure 1). One was formed by the *AP2* orthologs and genes involved in abiotic responses like *CBF1*, *SlCBF1* or *DREB2A* (Liu *et al.*, 1998; Weiss and Egea-Cortines, 2009; Jaglo-Ottosen *et al.*, 1998). A second distinct clade contained the *AINTEGUMENTA LIKE (AIL)* genes, *Brassica napus BABYBOOM* (*BBM*) and *PLT1* and 2. The genes *PhANT* and *AmANT* were inside a clade containing the *ANT* gene from *Nicotiana tabacum* (Rieu *et al.*, 2005), and other *ANT* genes from Solanaceae like *Solanum lycopersicon* and *Nicotiana benthamiana* as closest homologs while genes of Santalales like *Phoradendron serotinum* and *Comandraum bellata* clustered together. The closest clade contained the *ANT* from *Arabidopsis* that clustered with *ANT2* from *Brassica*. The clade containing *PhANT* and *AmANT* was clearly separated from the *AP2* group comprising *AP2*, the *Petunia PhAP2A* (Maes *et al.*, 2001) and the *Antirrhinum* paralogs *LIPLESS 1* and *2* (*LIP1* and 2)(Keck *et al.*, 2003). These results indicate that the *PhANT* gene isolated and used for further analysis displays a high degree of homology to *AtANT* and is a likely ortholog.

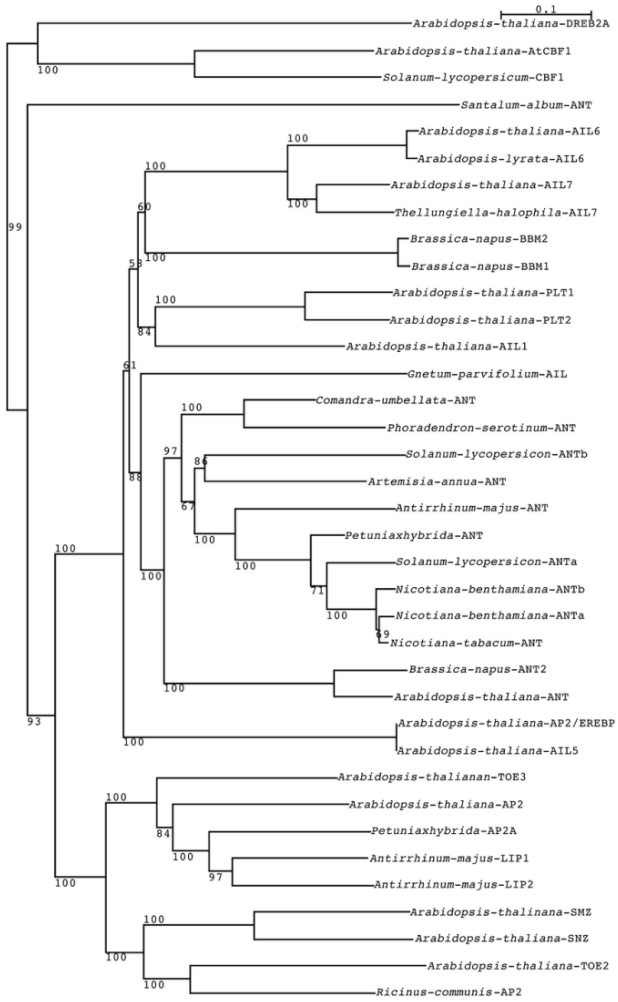

Figure 1. Phylogenetic tree of *APETALA2* related predicted proteins.
Numbers on branches correspond to number of bootstraps supporting them.

Differential effect of *PhANT* RNAi in limb and tube of *Petunia*

Although *AP2* transcription factors are involved in activation of cell division and play a role in stem cell maintenance and patterning in *Arabidopsis* (Wurschum *et al.*, 2006), we obtained kanamycin resistant plants in *Petuniaxhybrida Mitchell* containing an RNAi construct targeting the endogenous *PhANT* (*PhRNAi::ANT)* indicating that a certain down regulation of *PhANT* by *PhRNAi::ANT* is compatible with *in vitro* regeneration in this cultivar. We cannot rule out that the *calli* with stronger initial expression of the transgene did not regenerate. All the kanamycin resistant T1 plants, obtained by selfing the T0 generation, showed flowers that appeared smaller than non-transformed control plants. A careful inspection of the segregating populations revealed three distinct phenotypic classes, one displaying very small flowers, a second one with intermediate size and a third that displayed wild-type flowers (Figure 2a, 2b and 2c). Plants with wild-type flowers turned out to be those that had segregated out the *NPTII* gene and did not harbour the T-DNA insertion. We used these non-transgenic siblings as control plants for further experiments.

Figure 2. Phenotype of *Petunia* flowers with silencing of *PhANT* gene or overexpression of *AtANT*. Effect of the silencing of *PhANT* gene in (a) limb of *Petunia hybrida*, non-transformed (b) intermediate sized line and (c) small sized line. Side view showing the tube of (d) non-transformed on the left and smaller line on the right. (e) Comparison between wild-type (on the left) and *35S::AtANT* (on the right).

In the smallest flowers, we observed a significant reduction of 40% in the diameter of the limb compared to the wild-type phenotypes (p=0.0) (Table 1). Tube length reduction was smaller (13%) albeit significant (Figure 2d). Stamen length reduction was not significant while stigmas were significantly smaller. In contrast, sepal size was not affected (Table 1).

Table 1: Comparison of floral parameters between wild-type and transgenic lines.

	Corolla	**Tube**	**Stamen**	**Stigma**	**Sepal**
wild-type	36.8±2.3	43.1±2.8	35.5±2.8	38.1±1.4	12.1±1.3
***Petunia* RNAi-*ANT* medium**	28.8±0.4***	39.9±0.7	33.7±0.5	37.2±0.6	11.7±0.5***
% *Petunia* RNAi-*ANT* medium	-21.8	-7.5	-5.1	-2.6	-3.6
***Petunia* RNAi-*ANT* small**	21.9±0.8***	37.4±1.2	30.4±1.3	33.2±1.4	12.7±0.9***
% *Petunia* RNAi-*ANT* small	-40.5	-13.2	-14.3	-12.9	+5.0
Petunia* 35S::*ANT	52.5±3.6**	48.5±3.5	43.4±2.7**	46.0±1.9**	13.2±1.4***
% *Petunia* 35S::*ANT*	+42.7	+12.6	+9.2	+22.4	+20.5

Values correspond to averages (mm) ± standard error (n=20). P-values are represented with * p<0.05, ** p<0.01 and *** p<0.001.

Overexpression of *AtANT* in *Petunia*

We produced 18 independent *Petunia* transgenic lines expressing the *AtANT* gene driven by the 35S promoter (Krizek, 1999). Transformed *Petunia* plants were selfed and T1 plants segregating positive for kanamycin had flowers that were significantly larger than non-transformed siblings (Figure 2e). Plants overexpressing the *AtANT* gene from *Arabidopsis* had larger floral organs. Flower limbs

showed a stronger phenotype with 44% bigger flower limb diameter as compared to non-transformed plants (p=0.01). At first sight tubes looked similar but careful analysis showed a small (12%) but significant increase in tube length (Table 1). Both stamen and style were also significantly bigger but size changes were small. Regarding the length of the sepal, the transgenic lines displayed non-significantly larger (20%) first whorl organs (Table 1).

As we had observed in the *PhRNAi::ANT* plants, overexpression of *AtANT* caused significant size changes in the petal limb and smaller albeit significant changes in tube length.

Changes in expression levels of *ANT* in *Petunia* expressing RNAi constructs and overexpressing *AtANT*.

In order to verify if the observed floral phenotypes were correlated with changes in the endogenous levels of *PhANT* expression, we analysed transcript levels by RT-qPCR. As the 35S promoter is known to be active in all aerial parts of the plant, but changes in organ size were not homogenous (Figure 2; Table 1), we analysed the expression of the gene *PhANT* in tube and limb of the *PhANT* RNAi lines. Plants expressing the *PhRNAi::ANT* construct showed a progressive decrease in gene expression of the endogenous *PhANT* that correlated with the size of the flower. We used the normal size siblings as control of wild-type *PhANT* gene expression (value 100%), as they corresponded to siblings that segregated without kanamycin resistance. Petal limbs of middle-sized flowers displayed significantly lower expression of *PhANT* (60%; p=0.0) compared to siblings with wild-type size flowers while the smaller flowers showed an even stronger down regulation to 25% compared to wild-type siblings (p=0.0) (Figure 3a). Surprisingly, we could not find significant differences in the down regulation of *PhANT* in the tubes of intermediate (96%; p=0.760) or small flowers (93%; p=0.895) (Figure 3b). Finally we analysed for the presence of the *AtANT* mRNA in plants that were positive for kanamycin and displayed larger flowers. We did not expect downregulation of the endogenous gene resulting from co-supression as the sequences of *AtANT* and *PhANT* are very different. The endogenous *PhANT* in the limb was indeed similar to that found in non-transgenic siblings with normal sized flowers. However, the expression of *AtANT* was close to 42 fold higher than the endogenous *PhANT* gene expression (Figure 3c). The expression levels were different in the tube as the expression levels of *AtANT* were roughly two fold higher than the endogenous *PhANT* levels (Figure 3d). The differences in gene expression levels of *AtANT*, correlate with the different phenotypic effects found in both petal regions.

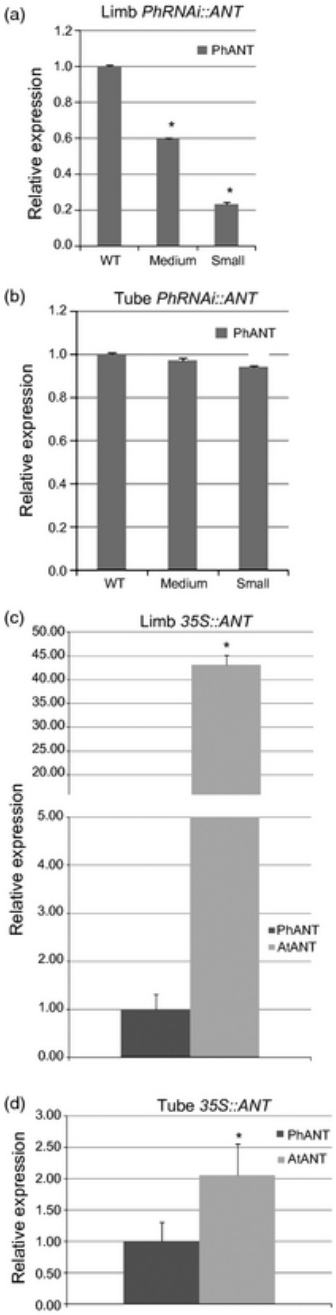

Figure 3. Expression of *AtANT* and *PhANT* in flowers of transgenic lines. Downregulation of *PhRNAi::ANT* gene compared to non-transgenic siblings (value = 1) in the (a) limb and (b) tube of transgenic lines *PhANT*. Expression of *AtANT* and *PhANT* in plants with overexpression of *AtANT* in (c) limb and (d) tube or *Petunia* flowers compared to the endogenous *PhANT*. *P*-values are represented with *<0.05.

Effects of modified *ANT* expression levels on cell size and morphology in *Petunia*

As both gain and loss of function of *ANT* in *Arabidopsis* affect organ size in a complex way regarding cell division and expansion (Krizek, 1999; Mizukami and Fischer, 2000), we measured cell area in three parts of the *Petunia* petal using scanning electron microscopy and image analysis (Figure 4).

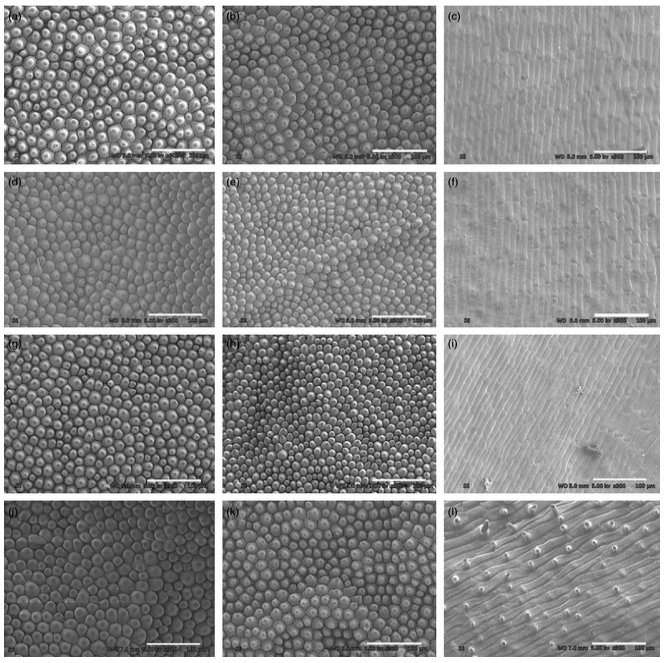

Figure 4. Cell size in *Petunia* transgenic lines. Comparison among the cell size in wild-type (on the top), the phenotypes obtained from silencing of *PhANT* gene, intermediate size (second row) and smaller size (third row); and the phenotypes of the overexpresion of *AtANT* (on the bottom). (a, d, g, j) are cells from the petal limb distal region. (b, e, h, k) correspond to cells from the petal limb proximal region. (c, f, i, l) cells from the petal tube.

In plants expressing *PhRNAi::ANT*, cells from the distal petal region were significantly bigger in small flowers (p=0.002) and intermediate size flowers (p=0.0) compared to non-transgenic sibling plants with an increase of 12% and 38%, respectively. In contrast, cells from the proximal part of the petal were significantly smaller in the intermediate size flowers (p=0.019) and small flowers (p=0.0) with a reduction of 9% and 26%, respectively, compared to non-transformed plants (Table 2). Tube cells were significantly smaller than in non-transformed plants in both intermediate size flowers (5%, p=0.035) and small flowers (10% p= 0.0086), indicating that the effect on petal tube length was the result of decreased cell expansion. Petal tube cell morphology was the typical expected for wild-type *Petunia* (Baumann *et al.*, 2007) in non-transgenic siblings. In contrast, plants with reduced *PhANT* expression lacked the typical protrusions (Figure 4 f,i), uncovering a possible effect of *PhANT* on petal tube epidermal cell differentiation.

Table 2: Cell size of *Petunia* flowers. Total number of cell measured for each organ/mutant = 100.

	Petal 1 (μm^2)	**Petal 2 (μm^2)**	**Tube (μm^2)**
wild-type	372.8±13.8	377.3±7.6	729.6±14.2
Petunia* 35S::*ANT	908.2±49.3***	416.2±10.7*	1932.9±47.2**
% *Petunia* 35S::*ANT*	+143.6	+10.3	+164.9
***Petunia* RNAi-ANT medium**	514.5±12.5***	341.7±7.2**	682.8±13.9*
% *Petunia* RNAi-ANT medium	+38.0	-9.4	-6.4
***Petunia* RNAi-ANT small**	416.6±9.4**	306.1±8.2***	656.2±27.2**
% *Petunia* RNAi-ANT small	+11.7	-18.9	-10.1

Values represent mean (μm^2) ± typical error. P-values correspond to *p<0.05, **p<0.01 and ***p<0.001

Differences in cell size reduction between the proximal region of the limb and tube in *PhRNAi*::*ANT* plants coincide with the differences observed in the size of these organs. Our data also indicate that the effect of *PhANT* silencing on cell size is not homogenous throughout the limb and tube.

In plants overexpressing *AtANT*, cells from the distal petal region (p=0.0), proximal petal region (p=0.03) and the tube (p=0.002) were significantly bigger than wild-type, increasing up to approx. 2.5 fold in the distal petal region and tube. We also found that like in wild-type plants, epidermal tube cells showed protrusions (Figure 4 l). Our results indicate a possible requirement of *PhANT* for the final stage of petal epidermal differentiation. We can conclude that overexpression of *AtANT* causes increased cell expansion in all regions analyzed. Our results indicate that the tube length increase is the result of increased cell expansion.

Overexpression of *Aintegumenta* in *Antirrhinum*

We obtained 15 independent lines of *Antirrhinum* resistant to kanamycin and positive for the *35S*::*AtANT* construct. The transgenic *Antirrhinum* plants had been developed using the commercial genetic background Vilmorin nain (www.vilmorin.com), a semi-dwarf bushy variety with extremely large flowers (Weiss *et al.*, 2012). We were able to identify two plants with flowers that exceeded the large size of this cultivar (Figure 5). The transgenic lines showed normal growth habit (Figure 5a). From two lines that showed extremely large flowers (Figure 5a), we were able to identify overexpression of the *AtANT* gene in flower limbs and tubes (Figure 5 b,c). All the flowers found were significantly larger than the original cultivar but the construct did not affect leaf area (Figure 5 d-g). *Antirrhinum* plants expressing *AtANT* were completely sterile as expected if we consider the experimental evidence from *Arabidopsis* (Mizukami and Fischer, 2000; Krizek, 1999). As the *Antirrhinum* flower has a more complex architecture than that of *Petunia*, we measured twelve floral parameters (Bayo-Canha *et al.*, 2007; Delgado-Benarroch., *et al.*, 2009b) in order to identify the parts of the flower with significant changes in size. Seven parameters were significantly increased, comprising tube length (118%; p=0.00018), bottom floral length (14%; p=0.041), petal width (53%; p=0.031), sepal length (97.5%; p=0.00091), ventral petal width (103%; p=0.00058), dorsal petal (78%; p= 0.0044) and palate (68%; p=0.0093) (Table 3). In contrast, top floral length, tube width, stamen and gynoecium size were not significantly affected by the 35S::*AtANT* expression. Changes in floral size in *Antirrhinum* were the result of increased tube length and petal width, while petal length was significantly increased but to a lesser extent.

Table 3. Comparison of floral parameters between wild-type and transgenic lines

	Tube length	Bottom petal length	Top petal length	Petal width	Sepal length	Tube width	Ventral petal width	Dorsal petal width	Stamen length	Gynoeci. length	Palate width
Wild-type	11.1±0.4	14.5±0.2	16.1±0.3	14.2±0.3	12 0±0.0	15.9±0.2	11.7±0.3	12.9±0.4	15 3±0.4	16.3±0.3	13.4±0.3
***Antirrhinum* 35S::ANT**	24 3±0.1***	21.7±0.6*	20.3±0.8	21.8±0.4*	23.7±0 3***	21.6±0.1	23.9±0.5***	22.9±1.1*	21 0±0.3	21.2±0.2	22 5±0.2**
% *Antirrhinum* 35S::ANT	118%	14%	26%	53%	97.5%	36%	103%	78%	37%	30%	68%

Values correspond to averages (mm) ± standard error (*n* = 20). *P*-values are represented with *<0.05, **<0.01 and ***<0.001.

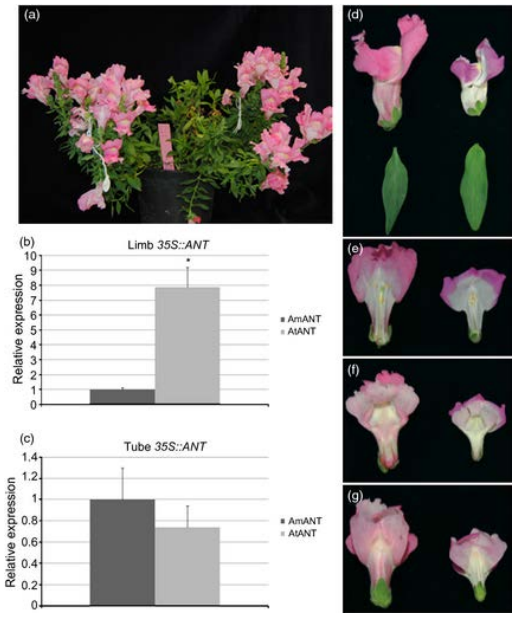

Figure 5. Expression of *AtANT* in transgenic *Antirrhinum* and phenotypes of *Antirrhinum* overexpressing *AtANT*. Transgenic *Antirrhinum* plant overexpressing *AtANT* (a). Expression of *AtANT* in limb (b) and tube (c) compared to the endogenous level of *AmANT* (value = 1). Floral and vegetative phenotypes of wild-type (on the right) compared to transgenic line of *Antirrhinum* overexpressing *AtANT* (on the left) flowers in lateral position and leaves (d) longitudinal section (e) frontal view (f) and dorsal position (g).*P*-values are represented with *<0.05.

Effect of *AtANT* on size in *Antirrhinum*

We measured the effects of *AtANT* overexpression on cell size by scanning electron microscopy. Cells from the distal region and proximal region of the limb, representing zones of conical and flat cells, were significantly bigger (p=0.0) than wild-type with a 2 fold and 1.7 fold increase, respectively (Figure 6, Table 4). Likewise the size of cells from the tube was 1.8 fold bigger (p=0.0). In summary, the effect of *AtANT* in *Antirrhinum* is a linear increase in cell size in all petal regions analysed. Cell size changes can explain larger petal organs.

Table 4. Cell size of *Antirrhinum* flowers.

	Region 3 (µm^2)	**Region 8 (µm^2)**	**Tube (µm^2)**
wild-type	1569.3±30.8	1264.7±27.9	3867.7±125.7
***Antirrhinum* 35S::ANT**	3320.9±69.3***	2212.1±44.8***	6881.4±185.7***
% *Antirrhinum* 35S::ANT	+111.6	+74.9	+77.9

Total number of cell measured for each organ/mutant = 100. Values represent mean (µm^2) ± typical error. P-values correspond to *p<0.05, **p<0.01 and ***p<0.001

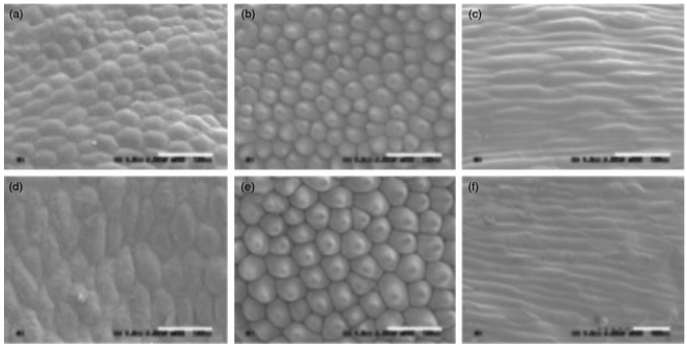

Figure 6. Cell size in *Antirrhinum*. Comparison between cell size of wild-type (on the top) and transgenic line with overexpression of *AtANT* (on the bottom) (a) and (d) cells from the proximal region of the petal (b) and (e) cells from the distal region of the petal limb (c) and (f) cells from the petal tube.

Overexpression of *AtANT* in *Arabidopsis* causes modifications of lateral organ size partly by changes in cell division and expansion (Mizukami and Fischer, 2000; Krizek, 1999). However, the main conclusion we could draw from the parallel experiments in *Petunia* and *Antirrhinum* are a major effect of *ANT* overexpression on cell expansion.

Differential effects of *AtANT* on endogenous *ANT* in *Petunia* and *Antirrhinum*

We found recently that the structure of gene regulatory network motifs can change during ontogeny (Manchado-Rojo *et al.*, 2012). We wondered if the detected differences between limb and tube could also be reflected in changes in the *ANT* network topology. We compared the levels of the endogenous *PhANT* and *AmANT* between limb and tube in *Petunia* and *Antirrhinum*. We found that during the stage of flower opening, the levels of *ANT* transcription tended to be lower in the limb compared to the tube in both flowers (Fig. 7 a). However, these differences in expression levels were significant in *Antirrhinum* (down to 0.312, p= 0.004) but not in *Petunia* (down to 0.509, p=0.142).

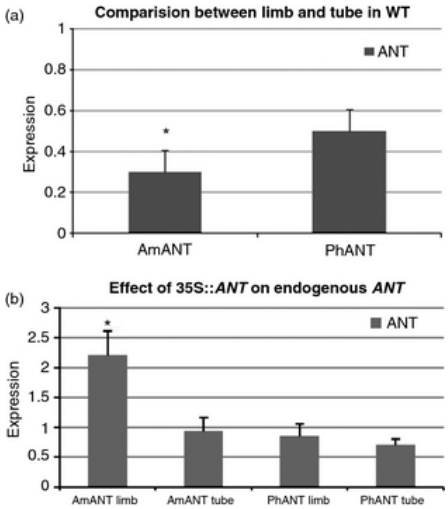

Figure 7. Levels of endogenous *AINTEGUMENTA* in limb and tube in non-transgenic and transgenic plants. Relative differences in endogenous *AmANT* and *PhANT* expression in limb (value = 1) and tube (a). Effect of *35S*::*AtANT* on the endogenous expression of *AmANT* and *PhANT*. Comparison of expression levels between limb and tube in non-transformed flowers (value = 1) (b). *P*-values are represented with *<0.05

As the results obtained in *Petunia* and *Antirrhinum* show a trend but are not identical, we suspected that the *ANT* regulatory network could be different. We compared the endogenous levels of *PhANT* in non-transgenic and transgenic lines overexpressing *AtANT*. These data should identify if *ANT* has some type of self-regulatory network motif, as positive or negative autoregulation. We would not expect RNAi-based co-supression of the endogenous *ANT* genes that display dissimilar DNA sequence as compared to *Arabidopsis*. In *Petunia*, *PhANT* was not regulated in the limb as a result of *AtANT* expression (0.802, p=0.386; Figure 7b). In contrast in *Antirrhinum*, *AtANT* caused a significant up-regulation of the endogenous *AmANT* in the limb (2.2 fold p=0.0). The *AtANT* expression did not affect the levels of *PhANT* or *AmANT* in the tube. Our data shows that *Petunia* and *Antirrhinum* share a common structure of regulatory network of the *ANT* gene in the tube where it is not subject to positive autoregulation. However a positive autoregulation exists in the limb of *Antirrhinum* but not in *Petunia* showing here an evolutionary divergence concerning the *ANT* regulatory network.

Discussion

The *ANT* gene belongs to the *AP2* family of plant-specific transcription factors. The *AP2* gene family comprises two major clades, one defined by *AP2* and a second one by *ANT* (Kim *et al.*, 2006; Shigyo *et al.*, 2006). The *ANT* lineage is present in the moss *Physcomitrella patens* but not in the green algae *Chlamydomonas reinhadtii*, indicating that it is a group formed later in evolution (Shigyo and Ito, 2004). The gene *ANT* from *Arabidopsis* has been described as a gene involved in growth control (Krizek, 1999; Mizukami and Fischer, 2000). In spite of its potential in *Arabidopsis*, its use as a biotechnological tool has not been established. We had previously cloned and characterized the *ANT* ortholog from *Antirrhinum*, *AmANT* (Delgado-Benarroch *et al.*, 2009a). It shows a heterochronic change in gene expression and its upregulation in inflorescences and young floral buds of the *formosa* mutant compared to wild-type could explain the larger flowers of this mutant. This made it especially attractive as a candidate gene to study modification of floral size in ornamentals. In this work we have cloned an *ANT* homolog from *Petunia* and we have performed a functional study in *Petunia* and *Antirrhinum* by modification of gene expression.

Loss of function of *ANT* in *Arabidopsis* causes a general decrease in lateral organ size and sterility (Mizukami and Fischer, 2000; Krizek, 1999). The overexpression of *AtANT* in *Arabidopsis* has three major phenotypic effects. Firstly at the macroscopic level, it causes a general increase in lateral organ size. Contrasting evidence shows increased petal size caused by cell division (Mizukami and Fischer, 2000) or cell expansion (Krizek, 1999). Finally *35S*::*AtANT* causes plant sterility (Mizukami and Fischer, 2000; Krizek, 1999). Thus we expected three possible effects of *ANT* overexpression in *Petunia* and *Antirrhinum*.

Overexpression of *AtANT* in *Petunia* and *Antirrhinum* does not cause similar macroscopic phenotypic changes. We obtained clear results of increased limb sizes in *Petunia*, and a general increase in petal limb and tube in *Antirrhinum* but the petal tube was less affected in *Petunia*. Genetic separation of limb and tube are found in the genus *Petunia*, where at least five loci control tube size (Galliot, Hoballah, *et al.*, 2006; Stuurman *et al.*, 2004). Differences in limb *versus* tube size are considered part of its pollination syndrome and adaptation to day and night pollinators (Galliot, Stuurman, *et al.*, 2006). Additional evidence for a distinct control of limb and tube have been obtained by misexpression of *EXPANSIN* showing a preferential effect on petal limb size whereas tube length is less affected (Zenoni *et al.*, 2004; Zenoni *et al.*, 2011). These results coincide with our data obtained in the Mitchell background, indicating that at least part of the floral size control in the genus *Petunia* differs between limb and tube.

In the *Antirrhinum* genus, natural variation of floral size shows increases and decreases of overall floral size but it does not display this clear separation of tube and limb as in *Petunia* (Feng *et al.*, 2009). Our results show that in *Antirrhinum*, *AtANT* causes increased growth as a result of larger tube and petal width, and importantly it does not cause a differential growth of the limb versus the tube. We can conclude that misexpression of *AtANT* does modify floral size, but the exact outcome might be constrained by the genomic environment where the gene is expressed, as the phenotypic spaces obtained resemble those seen in the genus.

We found that changes in the expression levels of *PhANT* by *RNAi* or overexpression of *AtANT* caused a complex cellular phenotype. As changes in cell size were larger than those observed in tube size we conclude that a compensation mechanism could take place in the *Petunia* tube. Compensation mechanisms are a cellular phenotype described in *Drosophila* and found also in plants where changes in genes affecting cell division would cause increases or decreases in cell expansion that would balance to some extent the effect on organ size (Neufeld *et al.*, 1998). This compensation mechanism has been extensively studied in leaf, ovule and flower development (Delgado-Benarroch *et al.*, 2009c; Ferjani *et al.*, 2007; Horiguchi and Tsukaya, 2006; Truernit and Haseloff, 2008). One current view is the existence of an underlying coordination between cell division and expansion in order to maintain a certain organ size, giving as a result a special resilience of an organ or organ region to changes in their proportions.

Overexpression of *AtANT* in *Arabidopsis* causes increased organ size as a result of cell division and cell expansion (Krizek, 1999; Mizukami and Fischer, 2000). In the *formosa* mutant of *Antirrhinum* a heterochronic effect is observed where *AmANT* is overexpressed in petals that are larger than wild-type as a result of increased cell division (Luciana Delgado-Benarroch, Causier, *et al.*, 2009). This

indicates that *ANT* might well function enhancing the cellular process involved in growth at a certain developmental window and overexpression at later stages of petal development also causes cell expansion. As we found a major effect on cell expansion in *Petunia* and *Antirrhinum*, we might conclude that *AtANT* is a general gene suitable to modify lateral organ size, by a combination of cell division and expansion, as we cannot rule out increased cell division at a certain point.

We found contrasting phenotypes concerning fertility in plants overexpressing *AtANT*. While *Petunia* was fertile, *Antirrhinum* showed complete sterility, as observed for *Arabidopsis* plants expressing *35S*::*AtANT*. From an industrial perspective, having transgenic plants that are sterile could be an asset, as environmental issues of horizontal gene transfer can be ruled out. Furthermore the horticultural industry is heavily based on vegetative propagation from shoots and vegetative cuttings (Hartmann and Kester, 1975). Thus transgenic lines can be multiplied at will.

As *ANT* is a gene with multiple functions, and the phenotypes found in *Petunia* and *Antirrhinum* share some common features, we analysed the differential levels of *ANT* in tube and limb and the effect of *AtANT* expression on the endogenous *ANT*. These experiments should shed light on the structure of the *ANT* regulatory network. First, it is surprising that in both *Petunia* and *Antirrhinum* the endogenous *ANT* expression tended to be lower in limb compared to tube. These data give some molecular support to the studies on petal size in *Petunia* and *Antirrhinum* showing differences between these two organ compartments (Stuurman *et al.*, 2004; Hermann and Kuhlemeier, 2011; Galliot, *et al.*, 2006; Delgado-Benarroch, *et al.*, 2009c). Additional support for a difference between petal limb and tube were found because the endogenous *ANT* expression in the tube of *Petunia* and *Antirrhinum* showed no changes as a result of expression of *AtANT*. However, the differences in positive autoregulation found in the *Antirrhinum* petal limb indicates that the *ANT* regulatory network (Alon, 2007) might differ between organ regions. Overall our data show that petal limb and tube might be considered separate organs in terms of genetic modification when it comes to size.

Altogether our data validates *ANT* as candidate gene to modify floral size in ornamental species. Although orthology can predict to a large extent the degree of conservation beyond the biochemical function, the annotation of single genes validated to modify biological functions in a predictable way is an on going effort in biology that is required to have universal biotechnological tools. Obtaining both larger and smaller flowers has been a major aim in breeding of *Petunia* and other ornamentals (Weiss *et al.*, 2006). The *ANT* gene might be seen as a potential target to obtain gain and loss of function alleles. Non-transgenic approaches like TILLING (Till *et al.*, 2003) or emerging technologies

like TALEN nuclease mediated genome engineering (Gaj *et al.*, 2013) could deliver new cultivars with modified floral size based on *ANT* alleles.

Experimental Procedures

Plant material and transformation

Seeds of *Petunia x hybrida* line Mitchell and *Antirrhinum majus nana* were surface-sterilized and sown on Murashige and Skoog medium (www.duchefa.com) solidified with 4g/L of Phytagel (www.sigmaaldrich.com) and were placed on growth chambers under a photoperiod of 16/8 hours of light/dark and 25ºC/18ºC temperature.

We carried out *Antirrhinum* transformation following the protocol based on hypocotyls previously described (Manchado-Rojo *et al.*, 2012). For *Petunia* we used leaf disks as described (Horsch *et al.*, 1985).

Plants corresponding to primary transformants (T0) and selfed plants (T1) were genotyped for presence of the *NPTII* gene by PCR. A positive control was performed using the gene *UBIQUITINE* (*UBI*) for all reactions to rule out a negative result caused by low quality DNA. Plants positive for *UBI* and negative for *NPTII* were considered as non-transgenic in T0 or as plants segregating out the transgenic construct in T1 after self pollination. We obtained a total of 20 lines for *PhRNAi::ANT*, 18 for *35S::AtANT* in *Petunia* and 2 for *35S::AtANT* in *Antirrhinum*. We genotyped ten T1 plants per line of all the lines obtained. Floral, cellular and molecular phenotypes correspond to the average of all the flowers of all the lines ordered by phenotype (large, medium or small for *Petunia*, and transgenic and non-transgenic for *Antirrhinum*).

Aintegumenta cloning and vector construction

We amplified a fragment of 198bp (ANT for 5'-GGAAAAGTTGGCTGGAAACA-3' and ANT rev 5'-ACTCTGCCTGCTGGTGAATT-3') from *Petunia* Mitchell genomic DNA, using the primers based on the tomato *ANT* coding sequence. The corresponding PCR fragment was reamplified with primers containing attb1 and attb2 specific sequences in order to obtain recombination ready fragments in a two-step PCR. First, two *attb1* and *attb2* partial tails were introduced by PCR and a second amplification was performed with *attb1* and *attb2* external primers (supplementary Table S2). The corresponding product was recombined by GATEWAY technology into *pDONR221* vector (www.invitrogen.com), using BP recombinase. The resulting *pDONR::PhANT* was further recombined with LR recombinase into the *pHELLSGATE12* vector (Helliwell and Waterhouse, 2003). Correct orientation and presence of two fragments in opposite direction was detected by PCR as described in

the www.agrikola.org protocol. We used the sequence obtained from *Petunia* to perform RACE PCR and clone 1.2 Kb of *PhANT* cDNA (Genebank accession KF754796). Overexpression of *ANT* was performed using the construct from *Arabidopsis* pCGN1547 35S::*ANT* (Krizek, 1999).

Phylogenetic analysis

Phylogenetic analysis was performed using CLUSTALX for alignment. Aligned sequences (Figure S1) were used for tree construction with the PHYLIP NJ algorithm (Larkin *et al.*, 2007) and trees were rendered with NJ plot (Perrière and Gouy, 1996). We confirmed the phylogenetic analysis using an online pipe (http://www.phylogeny.fr/version2_cgi/index.cgi) (Dereeper *et al.*, 2010; Dereeper *et al.*, 2008). The pipeline comprises a sequence alignment set for maximum accuracy with MUSCLE (Edgar, 2004). Aligned sequences are automatically curated from poorly aligned positions and blocks with Gblocks (Castresana, 2000). The phylogenetic analysis is performed by PhyML that uses a Near Neighbour Interchange algorithm (Guindon and Gascuel, 2003) and trees are rendered by Treedyn (Chevenet *et al.*, 2006). Percentages of protein similarity were identified using the Needleman-Wunsch algorithm implemented in the NEEDLE program (www.ebi.ac.uk).

Measurements

To determinate quantitative differences in floral parameters describing floral size, we measured in *Petunia* sepal, petal, stamen and style length. For petal size we measured the diameter of the limb, the length of the tube from its inception till the tilt of the tube into the limb. Stamen and style length were measured from the bottom of the flower. For *Antirrhinum*, the floral parameters measured were tube length, lower length, petal height, sepal length, tube width, upper (total) length, lower petal width, upper petal width, stamen length and gynoecium length (Delgado-Benarroch, *et al.*, 2009a). All the measurements were performed with a scalimeter when the flowers were fully open.

Scanning electron microscopy

In *Petunia* the petal was separated in limb and floral tube with a scalpel blade. Limbs of *Petunia* were further divided into a distal outer zone and a proximal zone near the tube in order to measure the cell area (Figure S1). For *Antirrhinum* we measured the cells from the region 3 and 8 of the limb corresponding to proximal and distal cells from the upper petal (Delgado-Benarroch *et al.*, 2009a).

Petal tissue was sliced into 5 mm^2 approximately. Sections were washed in wash buffer pH 7.2 (112mM NaH_2PO_4 and 288mM Na_2HPO_4), fixed overnight in glutaraldehyde 2%, dehydrated in a

graded ethanol series, placed in acetone 100% and finally dehydrated by critical point drying with liquid CO_2 (Delgado-Benarroch *et al.*, 2009a).

In order to analyse the cell size we measured the area of 100 cells from the limb and the tube, using the free software ImageJ (http://rsbweb.nih.gov/ij/). The data showed non-normal distributions, therefore they were analysed with the Kruskall-Wallis Test.

RT-qPCR

Total RNA was isolated with the NucleoSpin RNA plant kit (MACHEREY-NAGEL, www.mn-net.com) containing DNAase. The concentration of RNA was measured spectrophotometrically. We used 1 µg of total RNA in order to synthesize the first strand cDNA using the Maxima™ First Strand cDNA Synthesis Kit (Fermentas, www.fermentas.com).

Real-Time PCRs were performed in a Stratagene MX3000P QPCR system (Agilent Technologies, www.home.agilent.com) using a SYBR Green based PCR assay with ROX as reference dye (Brilliant II SYBR Green QPCR Master Mix. Stratagene, www.genomics.agilent.com). Primers were designed using PCREfficiency (supplementary Table S2) (Mallona *et al.*, 2011).

We used three biological replicas and two technical replicas for each sample. We obtained takeoffs and efficiency values from raw data (Mallona *et al.*, 2011) and computed differences in gene expression analysis as described previously using randomized group-wise statistical comparison using the REST program (Pfaffl *et al.*, 2002).

For relative quantification we used as reference genes the *RIBOSOMAL PROTEIN SAND 13* in the case of *Petunia* (Mallona *et al.*, 2010) and *UBIQUITIN* for *Antirrhinum*. The takeoff values of the genes *UBI*, *RSP13*, *AmANT* and *PhANT* were similar enough (supplementary Table S3) and stable to give reproducible results and be mathematically accepted by the REST program.

Statistical analysis

We used the R package for statistical analysis (www.r-project.org) or the StatGraphics programme, to perform analysis of variance. Data showing non-normality were analysed using the Kruskal-Wallis test.

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Supporting Information

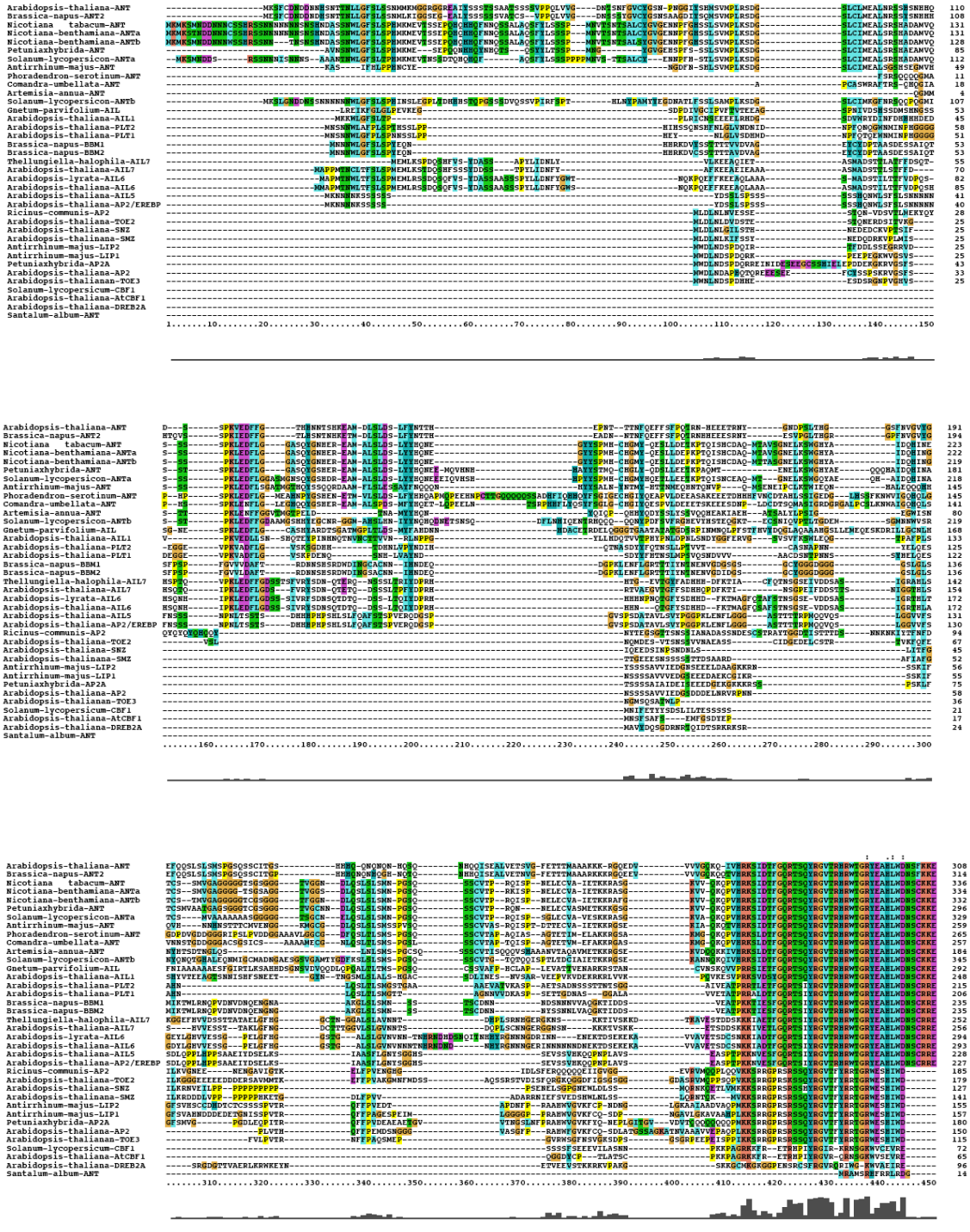


Figure S1: Multiple alignments of *AP2* and *ANT* predicted protein.

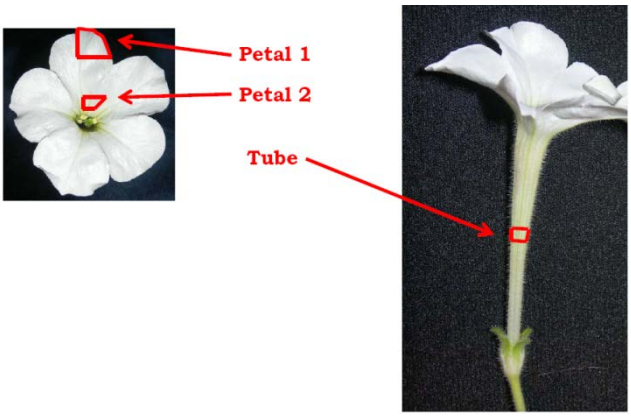


Figure S2: *Petunia* petal regions used to analyse effects of *ANT* expression on cell division and expansion.

Table S1: Accession numbers of genes used for phylogenetic analysis

Petunia x hybridaANTAHC98702.1; At4g37750 AtANT;A.majusANT KF975389;Nicotiana tabacum AY461432.1-NICOTIANA-ANTLIKE; FJ542318.1-PHORADENDROM-SEROTINUM-ANTLIKE; FJ542317.1-Comandra-umbellata-ANTLIKE; GQ468547.1-Artemisia-annua-ANT; DQ211970.1-Brassica-napus-ANT2; XM_002530201.1-Ricinus-communis; AB297493.1 Gnetum-parvifolium; XM_002871378.1-*Arabidopsis*-lyrata-ANTLIKE6; NM_125949.6-*Arabidopsis*-thaliana-AIL7; NM_001125733.1- *Arabidopsis*-thaliana-AIL6; NM_103997.3 *Arabidopsis*-thaliana-PLT2; NM_112975.2-*Arabidopsis*-thaliana-PLT1; GI:28894444-AmLIPLESS2; GI:28894442-AmLIPLESS1; GI:5081554-PhAP2A; At5g67180-TOE3; At4g36920g-APETALA2; At2g39250-SNZ; At1g72570-AIL1; At5g57390-AP2/EREBP; AF317904-Brassicanapus BABY BOOM1; AF317905-Brassica napusBABY BOOM2; At1g79700-AP2 like; At5g05410-DREB2A; AT4G25490-AtCBF1; NP_001234123-Solanum licopersiconCBF1;Solyc02g092050.2.1Solanumlycopersicon AINTEGUMENTA-a; Solyc04g077490.2.1Solanumlycopersicon AINTEGUMENTA-b; NbS00010330g0101.1Nicotianabenthamiana AINTEGUMENTA-a; NbS00030515g0004.1Nicotianabenthamiana AINTEGUMENTA-b

Table S2: List of primers used for PCR

PRIMER	SEQUENCE
PhANTfor	5’-ATGAAGTCTATGAATGATGATAAC-3’
PhANTrev	5’-TCATGCATCATTCAAAGCTGC-3’
PaxilarysANTfor	5’-TAATGATTTGCAATCTTTGAGC-3’
PaxilarysANTrev	5’-CAACATCATATGCTTCAGC-3’
PhSAND13 for	5’-CTTACGACGAGTTCAGATGCC-3’
PhSAND13 rev	5’-TAAGTCCTCAACACGCATGC-3’
AmANTfor	5’- TTGCTGCAATCAAGTTCAGG-3’
AmANTrev	5’-CAATGCCAATCAAATCATGC -3’
AmUBIQfor	5’-GCCGATGGAAGTATATGTTTGGACATC-3’
AmUBIQrev	5’-CTAACTTTGCGGTTATAATCTCGTTTA-3’
AtANTfor	5’-TCTAGTAACACACTCTTGTCTGG-3’
AtANTrev	5’-ATCAGCCCAAGCAGCGAAAAC-3’
attb1	5’-GGGGACAAGTTTGTACAAAAAAGCAGGCT-3’
attb2	5’-GGGGACCACTTTGTACAAGAAAGCTGGGTA-3’
Attb1PhANTfor	5’-CAAAAAAGCAGGCTATGAAGTCTATGAAT-3’
Attb2PhANTrev	5’-CAAGAAAGCTGGGTATCATGCATCATTCAA-3’

Table S3: Takeoff values of different genes in RT-qPCR experiments

35S::ANT PETUNIA		
RPS13	Tube	18.95±1.9
	Limb	21.75±1.2
PhANT	Tube	25.85±1.5
	Limb	22.5±0.8
35S::ANT ANTIRRHINUM		
UBIQ	Tube	22.11±1.7
	Limb	22.78±0.4
AmANT	Tube	22.24±1.3
	Limb	22.78±2.6

General Conclusions

General Conclusions

Chapter 1:

- Melting point analysis of the 23S rDNA amplicon obtained by RT-qPCR is a useful tool for genotyping *Agrobacterium tumefaciens* and *Escherichia coli.*
- The protocol allows to test clones that fail to transform plants as a result of contaminated bacterial stocks.
- The codominant PCR markers amplify from both bacteria in mixed cultures, generating amplicons whose melting curves can be easily differentiated.
- This method should be more useful than *Agrobacterium* or *E. coli* -specific PCR markers.

Chapter 2:

- The gene *compacta (co)* plays a role for proper homeotic gene function of the B class downstream of *DEF*, affecting activation and maintainance of *DEF* expression. This was demonstrated by the fact that *def*^{nic} single mutants and *co def*^{nic} double mutants have similar levels of *DEF* and *GLO*, but the phenotypes analyzed are more extreme in the double mutant.
- There is a quantitative component in gene function of the homeotic genes *DEF* and *GLO.* Recognizable petal development is achieved with 11% transcription level in the B-loss of function alleles *compacta* and *deficiens*. However, these levels do not sustain full organ size with properly developed conical cells and scent production.
- The necessary thresholds of B function are not uniform over all petal regions as indicated by the fact that flowers of *def*^{nic}, a weak allele of B function gene *DEF*, and *co def*^{nic} double mutants, only lack the palate of the flower while other petal structures are formed.
- The positive autoregulatory loop is only part of the B function maintenance and a basal level of transcriptional activation is present for both *DEF* and *GLO,* at least at late stages of development. Positive autoregulation comprises organ specific components and may differ between *DEF* and *GLO. S*ince linear levels of translation rates are maintained during petal development for *DEF* and *GLO*, the changes in mRNA should translate into differing levels of protein.

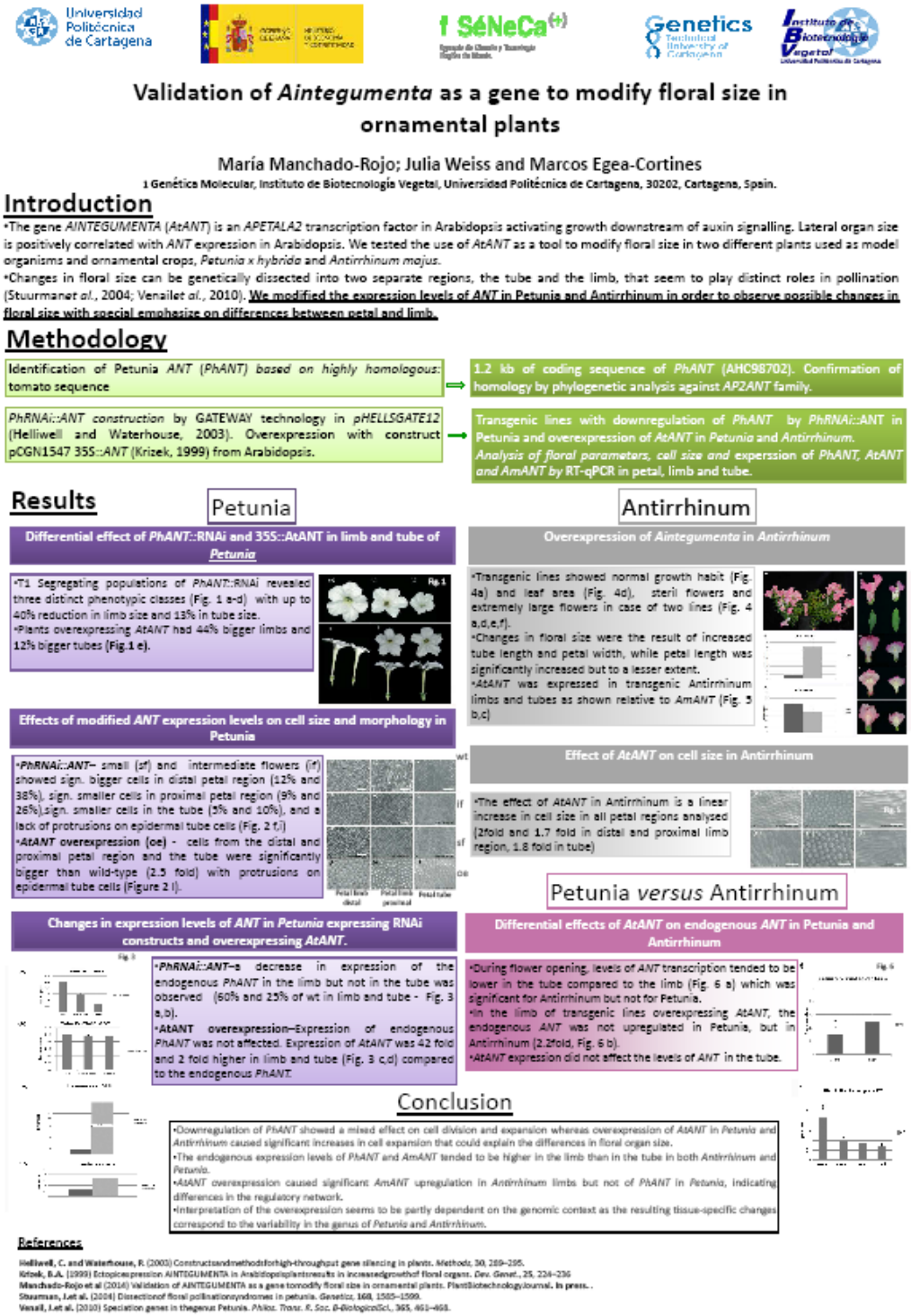
Chapter 3:

- Changes in *AINTEGUMENTA* gene expression in *Petunia* and *Antirrhinum* do not cause similar macroscopic phenotypic changes and the exact outcome might be constrained by the genomic environment where the gene is expressed, as the phenotypic spaces obtained resemble those seen in the genus.
- Changes in the expression levels of *PhANT* cause a complex cellular phenotype. As changes in cell size were larger than those observed in tube size we conclude that a compensation mechanism could take place in the *Petunia* tube in order to maintain a certain organ size.
- *AtANT* overexpression caused significant *AmANT* up-regulation in *Antirrhinum* limbs but not of *PhANT* in *Petunia*, indicating differences in the regulatory network of *ANT* between these two species.
- Differences in positive autoregulation were also found between petal limb and tube in *Antirrhinum*, indicating that the *ANT* regulatory network might differ between organ regions. Our data show that petal limb and tube might be considered separate organs in terms of genetic modification when it comes to size.
- Altogether our data validates *ANT* as candidate gene to modify floral size in ornamental species.

Annex I: Congresses

Annex I: Congresses

- Manchado-Rojo, M., Weiss, J. and Egea-Cortines, M. (2014). Validation of *Aintegumenta* as a gene to modify floral size in ornamental plants. XII Reunión Biología Molecular de Plantas. XII Reunión Biología Molecular de Plantas (Cartagena – Murcia, Spain)



Validation of *Aintegumenta* as a gene to modify floral size in ornamental plants

María Manchado-Rojo; Julia Weiss and Marcos Egea-Cortines
1 Genética Molecular, Instituto de Biotecnología Vegetal, Universidad Politécnica de Cartagena, 30202, Cartagena, Spain.

Introduction

•The gene *AINTEGUMENTA* (*AtANT*) is an *APETALA2* transcription factor in Arabidopsis activating growth downstream of auxin signalling. Lateral organ size is positively correlated with *ANT* expression in Arabidopsis. We tested the use of *AtANT* as a tool to modify floral size in two different plants used as model organisms and ornamental crops, *Petunia x hybrida* and *Antirrhinum majus*.
•Changes in floral size can be genetically dissected into two separate regions, the tube and the limb, that seem to play distinct roles in pollination (Stuurman*et al.*, 2004; Venail*et al.*, 2010). We modified the expression levels of *ANT* in Petunia and Antirrhinum in order to observe possible changes in floral size with special emphasize on differences between petal and limb.

Methodology

Identification of Petunia *ANT* (*PhANT*) *based on highly homologous*: tomato sequence ⇒ 1.2 kb of coding sequence of *PhANT* (AHC98702). Confirmation of homology by phylogenetic analysis against *AP2ANT* family.

PhRNAi::ANT construction by GATEWAY technology in *pHELLSGATE12* (Helliwell and Waterhouse, 2003). Overexpression with construct pCGN1547 35S::*ANT* (Krizek, 1999) from Arabidopsis. → Transgenic lines with downregulation of *PhANT* by *PhRNAi*::ANT in Petunia and overexpression of *AtANT* in *Petunia* and *Antirrhinum*. *Analysis of floral parameters, cell size and expersion of PhANT, AtANT and AmANT by* RT-qPCR in petal, limb and tube.

Results

Petunia

Differential effect of *PhANT*::RNAi and 35S::AtANT in limb and tube of *Petunia*

•T1 Segregating populations of *PhANT*::RNAi revealed three distinct phenotypic classes (Fig. 1 a-d) with up to 40% reduction in limb size and 13% in tube size.
•Plants overexpressing *AtANT* had 44% bigger limbs and 12% bigger tubes (Fig.1 e).

Effects of modified *ANT* expression levels on cell size and morphology in Petunia

•*PhRNAi::ANT*– small (sf) and intermediate flowers (if) showed sign. bigger cells in distal petal region (12% and 38%), sign. smaller cells in proximal petal region (9% and 26%),sign. smaller cells in the tube (5% and 10%), and a lack of protrusions on epidermal tube cells (Fig. 2 f,i)
•*AtANT* overexpression (oe) - cells from the distal and proximal petal region and the tube were significantly bigger than wild-type (2.5 fold) with protrusions on epidermal tube cells (Figure 2 l).

Changes in expression levels of *ANT* in *Petunia* expressing RNAi constructs and overexpressing *AtANT*.

•*PhRNAi::ANT*–a decrease in expression of the endogenous *PhANT* in the limb but not in the tube was observed (60% and 25% of wt in limb and tube - Fig. 3 a,b).
•AtANT overexpression–Expression of endogenous *PhANT* was not affected. Expression of *AtANT* was 42 fold and 2 fold higher in limb and tube (Fig. 3 c,d) compared to the endogenous *PhANT*.

Antirrhinum

Overexpression of *Aintegumenta* in *Antirrhinum*

•Transgenic lines showed normal growth habit (Fig. 4a) and leaf area (Fig. 4d), steril flowers and extremely large flowers in case of two lines (Fig. 4 a,d,e,f).
•Changes in floral size were the result of increased tube length and petal width, while petal length was significantly increased but to a lesser extent.
•*AtANT* was expressed in transgenic Antirrhinum limbs and tubes as shown relative to *AmANT* (Fig. 5 b,c)

Effect of *AtANT* on cell size in Antirrhinum

•The effect of *AtANT* in Antirrhinum is a linear increase in cell size in all petal regions analysed (2fold and 1.7 fold in distal and proximal limb region, 1.8 fold in tube)

Petunia *versus* Antirrhinum

Differential effects of *AtANT* on endogenous *ANT* in Petunia and Antirrhinum

•During flower opening, levels of *ANT* transcription tended to be lower in the tube compared to the limb (Fig. 6 a) which was significant for Antirrhinum but not for Petunia.
•In the limb of transgenic lines overexpressing *AtANT*, the endogenous *ANT* was not upregulated in Petunia, but in Antirrhinum (2.2fold, Fig. 6 b).
•*AtANT* expression did not affect the levels of *ANT* in the tube.

Conclusion

•Downregulation of *PhANT* showed a mixed effect on cell division and expansion whereas overexpression of *AtANT* in *Petunia* and *Antirrhinum* caused significant increases in cell expansion that could explain the differences in floral organ size.
•The endogenous expression levels of *PhANT* and *AmANT* tended to be higher in the limb than in the tube in both *Antirrhinum* and *Petunia*.
•*AtANT* overexpression caused significant *AmANT* upregulation in *Antirrhinum* limbs but not of *PhANT* in *Petunia*, indicating differences in the regulatory network.
•Interpretation of the overexpression seems to be partly dependent on the genomic context as the resulting tissue-specific changes correspond to the variability in the genus of *Petunia* and *Antirrhinum*.

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Stuurman, Let al. (2004) Dissectionof floral pollinationsyndromes in petunia. *Genetics*, 168, 1585–1599.
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- Manchado-Rojo, M.,Egea-Cortines, M. and Weiss, J. (2011).Functional analysis of pollination syndromes in *Petunia* and *Antirrhinum*. XI World *Petunia* Days (Lyon, Francia).

Petunia and *Antirrhinum* have opposite forms of evolution at least in one of the major traits involved in pollination syndromes. In *Antirrhinum*, single mutants affect floral size and in some cases organ regions (Delgado-Benarroch *et al.*, 2009b; Delgado-Benarroch *et al.*, 2009a). However, studies in natural populations show that evolution in the genus has occurred by changes in pleiotropic genes that affect both vegetative and reproductive development (Feng *et al.*, 2009). In contrast, in the *Petunia* genus, petal tube and limb have evolved as separate traits (Galliot *et al.*, 2006; Stehmann *et al.*, 2009; Venail *et al.*, 2010). Overexpression of Aintegumenta in *Arabidopsis* and tobacco causes overall increase of vegetative and reproductive organs, (Mizukami and Fischer, 2000). We have studied the effect of the *Aintegumenta* gene in transgenic plants of *Antirrhinum* and *Petunia* to identify if the opposite evolutionary history is the result of changes in gene function or genomic contest. *Petunia x hibrida* Mitchell plants expressing RNAi-*ANT* have smaller flower in a dosage dependent manner. Petal tube was roughly 86% of WT whereas limb expansion was decreased by nearly 40%. Thus loss of function of ANT resembles changes seen in other species of the genus. *Antirrhinum* plants overexpressing AtANT showed faster regeneration than those expressing RNAi DEF. Preliminary results indicate that floral size is globally increased. A comparison between *Petunia* and *Antirrhinum* flowers will be presented in the congress.

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  Genetic transformation is a cornerstone to obtain information of gene functions. We have developed an improved protocol for transformation and regeneration of *Antirrhinum majus*, obtaining a highly reproducible method that has yielded up to a high efficiency, close to 10% (the final results will be discussed in the congress). Several aspects affect transformation efficiency. We tested two lines, 165E and Vilmorin Nain and two different explants, leaf discs and hypocotyls from seedling of two and four weeks. As a proof of concept we transformed *A. majus* with a *pHellsgate12* construct expressing RNAi of the homeotic gene *Deficiens*. Two week old hypocotyls explant from the line Vilmorin Nain had the highest transformed rate. Putative transformants were tested by PCR using *NPTII* primers and by their phenotypes. The resulting plants showed classic phenotypes corresponding to hypomorphic alleles of *Def*,

which included sepaloid petals and sterile stamens and fell somewhere in strength between *deficiens*^{chlorantha} and *deficiens*^{nicotianoides}.

An improved protocol for transformation of *A. majus*

Manchado-Rojo, M.[1], Portero-Martinez, A.[1], Weiss, J.[1] and Egea-Cortines, M.[1]

[1]: Universidad Politécnica de Cartagena, Genetics. Instituto de Biotecnología Vegetal. 30203 Cartagena (Murcia)

ABSTRACT

Genetic transformation is a cornerstone to obtain information of gene functions. We have developed an improved protocol for transformation and regeneration of *Antirrhinum majus*, obtaining a highly reproducible method that has yielded up a high efficiency, close to 10%. Several aspects affect transformation efficiency. We tested two lines, 165E and Vilmorin Nain and two different explants, leaf discs and hypocotyls from seedling of two and four weeks. As a proof of concept we transformed *A. majus* with a pHellsgate12 construct expressing RNAi of the homeotic gene *Deficiens*. Two week old hypocotyls explants from the line Vilmorin Nain had the highest transformed rate. Putative transformants were tested by PCR using NPTII primers and by their phenotypes. The resulting plants showed classic phenotypes corresponding to hypomorphic alleles of *Def*, which included sepaloid petals and sterile stamens and fell somewhere in strength between *deficiens chlorantha* and *deficiens nicotianoides*.

MATERIALS AND METHODS

PLANT MATERIAL

We used two lines of *Antirrhinum majus*, 165E and Nain from Vilmorin. Seeds from both lines were surface sterilized by briefly rinsing first in 70% (v/v) ethanol, then in 1% (v/v) sodium hypoclorite for 10 minutes, and then washing with sterile distilled water five times. The seeds were germinated on Murashige and Skoog medium containing sucrose (25 g/l) and gelrite (2,5 g/l). We tested two-week old and four-week old hypocotyl explants.

AGROBACTERIUM-MEDIATED TRANSFORMATION

Agrobacterium tumefaciens strain EHA105 was used for transformation. We tested silencing of the *Deficiens* gene using the pHellsgate12 plasmid (Helliwell and Waterhouse, 2003) harbouring 207 bp encompassing the last 60 codons and 27 bp of the 3'UTR of *Def*, forming a hairpin (Figure 1). The complete Arabidopsis *Aintegumenta* cDNA was overexpressed using pCGN1547 (Krizek 1999). We used standard transformation procedures and modified media (Table 1).

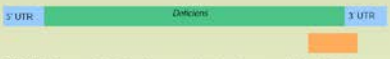

FIGURE 1: Construct of *Deficiens*. In orange shows the fragment used for the silencing.

TABLE 1: Composition of medium

SIM MEDIUM		CO-CULTIVATION MEDIUM		TRANS-CULTIVATION MEDIUM		ROOT MEDIUM	
Sodium citrate $2^{*}H_2O$	5.882 g/L	MS	4,4 g/L	MS	4,4 g/L	MS	4,4 g/L
Sucrose	20.0 g/L	Sucrose	25.00 g/L	Sucrose	25.00 g/L	Sucrose	25.00 g/L
Acetosyringone	500.0 µM	Gelrite	2.50 g/L	Gelrite	2,50 g/L	Gelrite	2.50 g/L
		Acetosyringone	100.00 µM	NAA	0,25 mg/L	NAA	0,20 mg/L
		NAA*	0,25 mg/L	Zeatine	2,0 mg/L	Cefotaxime	300 mg/L
		Zeatine	2,0 mg/L	Cefotaxime	300 mg/L	Kanamycin	50 mg/L
				Kanamycin	100 mg/L	Vancomycin	200 mg/L
				Vancomycin	200 mg/L		

* Naphtalene acetic acid

RESULTS

The results of the improved protocols for transformation of *Antirrhinum majus* are shown in table 2.

TABLE 2: Results of the transformation

EXPERIMENT	RESULTS IN PERCENTAGES
TYPE OF EXPLANT	
LEAF	3.10%
HYPOCOTYL	7.41%
AGE OF EXPLANT	
TWO WEEK	9.11%
FOUR WEEK	0%
ANTIRRHINUM LINE	
165E	1.39%
VILMORIN NAIN	8.28%
GENE	
DEFICIENS	4.50%
ANTEGUMENTA	5.28%

CONCLUSION

There are very important effects of the age and the type of explant used on transformation efficiency.

The phenotype of the silencing of *Deficiens* falls between *def*^{chlo} and *def*^{nic} (Figure 2).

Overexpression of *Aintegumenta* increased the number of shoots regenerated and obtained a higher number of transgenic calli in *in vitro* culture. Floral and vegetative phenotypes are currently being analyzed.

FIGURE 2: Silencing of the Deficiens gene in *A. majus*.

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Traditionally, cut flowers are transported in the horizontal way. This represent a problem for several species, between them we could stand *Antirrhinum majus* out, (Halevy and Mayak 1981; Tasaka*et al.* 1999 y 2001), since their stems have a strong negative gravitropism which

make the inflorescence stems curve upwards and this imply its commercial depreciation and troubles for marketing.
In *Arabidopsis thaliana* there are described seven genes related with the negative shoot gravitropism, they are the so call Shoot Gravitropism genes. This genes has been described by Fukaki (1996) (*SGR1*, *SGR2*, *SGR3*), Yamauchi (1997) (*SGR4*, *SGR5*, *SGR6*) and Fukaki (1998) (*SGR7*). We have identified the ortholog gene to *SGR4* in *A. majus* and the ortholog gene to *SGR5* in *P. hybrida*. So far, we are not able to identify more genes involved in shoot gravitropism due to the ESTs sequenced in this species are relatively not many.
A gene library is very important tool in inverse genetic. The cDNA libray is a representation of the genes from the genome of an individual, although they are not always in the same relation. There are genes which are 2000 times more expressed than other genes (Marra *et al.*, 1998). We have carried out a methodology with the aim to obtain a normalized cDNA libray. The analysis of gene expression quantification, to verify the normalization of the cDNA library, have been done by RT-qPCR and after that, the data were analyzed by REST program.

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We are interested in understanding the evolutionary and molecular basis of resistance genes in *Petunia hybrida* both for basic and breeding purposes. We have established a PCR strategy to identify oorthologs and paralogs from several gene families. We have used degenerate primers [1] to clone resistancce genes from the Mitchell line. We picked a total of 150 clones and have sequenced 26 clones. We have obtained several clones that correspond to LTR and Ty transposons and at least four resistance genes. These include putative orthologs of a TIR-NBS-LRR from *Solanumcaripense*, P-Loop-ATPase, one gene similar to the N-gene from tobacco (resistance to tobamovirus), and one R3-a like from potato. We are currently expanding the number of genes sequenced in order to have a set of *Petunia* sequences to be used for evolutionary and breeding purposes as markes and in transgenic experiments.

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  La existencia de información de secuencia en una especie, es determinante para poder llevar a cabo abordajes de genética inversa como TILLING, búsquedas de inserciones de transposones o ARN de interferencia. *Petunia hybrida* es una solanácea que ocupa el primer lugar en las ventas anuales de planta en maceta en Europa. Actualmente, solamente se conocen unas 7.000 ESTs secuenciadas. Hemos desarrollado un protocolo de normalizado y hemos realizado una genoteca ultranormalizada a partir de ARN de hojas, tallos, semillas, meristemos apicales, meristemos florales, primordios florales, y flores en tres estadíos de desarrollo. Mediante un análisis comparativo de la primera hebra de cDNA contra la genoteca normalizada para los genes actina, ciclofilina, GADPH, ubiquitina, PP2A, RAN3, tubulina y factor de elongación F muestra cambios en los niveles de expresión de los genes analizados, en los que la dispersión disminuye entre 10 y 100 veces. Se ha llevado a cabo una reacción de secuenciado de la genoteca por el sistema 454, y estamos analizando un total de 180.000 ESTs que deberían rendir más de 15.000 unigenes.

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