Molecular analysis of plant architecture in *Arabidopsis thaliana* using activation tagging

Antonio Chalfun Junior

Inflorescence of the *ds1-D* mutant plant

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Propositions

1. The use of activation tagging as a tool to discover gene function is very helpful to understand regulatory networks that control plant development (this thesis).

2. Negative regulation of a gene is correlated with positive regulation of another, and vice-versa (this thesis).

3. In science, one who walks alone may be the first to arrive, however, one who travels in a group is sure to go further.

4. A country without science is like a marriage without love.

5. Technology itself is not a nightmare but ignore it, it can become one.

6. For some a weed, for others a crop.

7. *"Libertas quae sera tamen"* (*Liberdade ainda que tardia* - Freedom even if it is late). Quote on the flag of the Minas Gerais State, Brazil.

8. There is not knowing more or less. There are different ways of knowing (*Não há saber mais ou menos. Há saberes diferentes,* Paulo Freire, Brazilian writer).

Propositions belonging to the Ph.D thesis:

"Molecular analysis of plant architecture in *Arabidopsis thaliana* using activation tagging"
by Antonio Chalfun Junior
Wageningen, April 21st, 2004

Molecular analysis of plant architecture in *Arabidopsis thaliana* using activation tagging

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Dit onderzoek is uitgevoerd binnen de onderzoekschool Experimentele Plantenwetenschappen (EPW).

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Proefschrift
ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van Wageningen Universiteit
Prof. dr. ir. L. Speelman
in het openbaar te verdedigen
op woensdag 21 april 2004
des namiddags te vier uur in de Aula

Chalfun-Junior, A. (2004). Molecular analysis of plant architecture in *Arabidopsis thaliana* using activation tagging. Ph.D. thesis, Wageningen University and Research Centre, Wageningen, The Netherlands. With summaries in English, Dutch and Portuguese.

ISBN: 90-8504-036-1

ABSTRACT

Plant development is one of the most important aspects of plant's life cycle that has extensively been studied at the morphological, genetic and molecular level. It is import for systematic and taxonomic classification, but also for applied agronomic reasons, because it affects the growth and cultivation leading to higher yield and quality of the product. The generation of genetic variants, like mutants may increase genetic pool and gives information about plant processes and their genetic control. Activation tagging is a new powerful tool to generate and identify new mutants, which emerged as an alternative for gene function analysis. This thesis reports the study on the molecular control of plant architecture, using mutants generated by an activation tagging-based approach in the model plant *Arabidopsis thaliana*. In addition, it also describes experiments that could explain why the low frequencies of mutants were obtained by T-DNA based activation tagging. Based on this comparison, the transposon-based activation tagging strategy was chosen and a screen for flower and silique mutants in a large *Arabidopsis* population yielded three gain-of-function mutants. These mutants were designated *downwards siliques1* (*ds1-D*), *needle1* (*ndl1-D*) and *twisted1* (*twt1-D*). In the *ds1-D* mutant, internodes are shorter and the lateral organs such as flowers are bending downwards. Further molecular and genetic studies on this mutant revealed that *DS1* is important to control petiole-blade boundary in *Arabidopsis* petals. In the *ndl1-D* mutant, the normal formation of valve tissues is altered, resulting in a pin-like structure that replaces the two fused carpels of the wild type pistil. The results suggest that *NDL1* is involved in normal carpel development, in which auxin distribution plays an important role. In the third mutant, *twt1-D*, the overexpression of *TWT1* led to twisting of all organs, which is most pronounced in siliques. This phenotype and the expression pattern of the gene suggest that *TWT1* is involved in proper vascular tissue development in *Arabidopsis*. These studies demonstrate the power of activation tagging and it gains valuable knowledge about the molecular networks that control plant development.

Keywords: *Arabidopsis thaliana,* activation tagging, T-DNA, transposon, mutants, enhancer, DNA methylation, plant architecture, development, forward/reverse genetics, lateral organs, flower, vascular tissue, HLH, transmembrane, transcription factors

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CHAPTER 1

General introduction

The last decade, a number of important topics have been extensively studied in the field of plant development. In these studies, many molecular techniques have been created to increase the understanding of the plant's life cycle. These technologies can be used to improve important plant characteristics, such as yield and resistance to pests and diseases. In this short introduction, the work described in this thesis will be related to ongoing work in the field of plant development, with an emphasis on plant architecture in the model plant *Arabidopsis*. The novel technologies used to generate mutants will be briefly compared to other state-of-art methods. Finally, the scope of the thesis will be presented and the main conclusions of the included chapters will be highlighted.

Plant architecture

Plant architecture involves many aspects that are under strict genetic and molecular control. Moreover, it is also regulated by environmental factors, such as light, temperature, humidity and nutrient levels. All these external and internal factors together, define the shape of the adult plant. An adult plant is composed of highly organized organs, which are derived from undifferentiated meristematic cells. These cells have passed through cell division, differentiation and growth under a precise genetic control, ultimately establishing the final phenotype of the plant. The various steps in plant development involve each a unique and complex set of genes that are switched on and off in time.

Botanists have used plant architecture characteristics for a long time as criteria for systematic and taxonomic classification. However, plant architecture is also extremely important for agronomic reasons. It influences e.g., the suitability of the cultivation leading to better yield. Thus, a better understanding of the plant architecture may lead to ways to control and modify agronomically important traits, like branching. Therefore, biologists, geneticists, agronomists and botanists have combined efforts to improve important aspects of plant architecture. Whether the desired crop can be directly used is trait dependent. In most of the cases, it is extraordinary laborious to study gene functions in every crop, therefore, studies on model plants (although a few crop plants have been used as well, like maize, rice and tomato) were initiated and have helped in understanding of the genetic basis of plant architecture. The main contemporary model is the flowering plant *Arabidopsis thaliana*.

Arabidopsis history

Arabidopsis thaliana (L.) Heynh. is a small weed that belongs to the mustard family, *Brassicaceae*. It is found naturally in temperate regions from Asia, Europe and

North Africa, but it was also introduced in other areas like North America and Australia. It has been the subject of deep research for a long time, as the first report using it as model plant dates back to 1907, in Frieddrich Laibach's PhD thesis, at the University of Bonn, Germany (Somerville and Koornneef, 2002). However, the widespread adoption as a model plant came much later in 1980 (Meyerowitz and Pruitt, 1985) and during the last decade its use has exponentially increased in studies related to all aspects of the plant life cycle. Its popularity as model system is attributed to its short generation time (about 8 weeks from sowing to harvesting mature seeds), small genome (125Mbp) and high gene density, small plant size (15-20cm in height), autonomous self-fertilisation, abundant number of seeds (about 3000-5000 seeds per plant) and easy transformation system (Meinke et al., 1998; *Arabidopsis* Genome Initiative, 2000). All these advantages have made this plant species the beloved of scientists all over the world. In 2000, its genome was completely sequenced (*Arabidopsis* Genome Initiative, 2000), and about 25.000 genes were annotated. For most of them, functions of these genes need to be unraveled, being the crucial point of the "-omics" era for *Arabidopsis*. At this moment, an impressive amount of data are being produced by high throughput methods, e.g., large scale expression studies (microarrays), shading light on the unknown gene functions. Even though, they still require validation and studies to confirm the hypotheses or gene functions, making use of mutants. Information about *Arabidopsis* is mostly available at three websites: at TAIR (www.arabidopsis.org); at GARNET (http://www.york.ac.uk/res/garnet/garnet.htm) and at MIPS (http://mips.gsf.de/proj/thal/proj/thal_overview.html). In table 1, a few other important websites where information about *Arabidopsis* can be found are given.

Forward and reverse genetics: the generation of mutants

The completion of the *Arabidopsis* genome gave a powerful support for the development of reverse genetics approaches to determine gene functions. Reverse genetics begins from the gene sequence and tries to generate a mutant phenotype. On the other hand, forward genetics (the traditional genetic approach) starts with a mutant phenotype, but subsequently, gene cloning is needed to say something about the nature of the gene. One way to directly link gene function to a phenotype is by loss-of-function due to a mutation in the gene. There are several ways to induce mutations and a very effective approach is random insertion mutagenesis, which can be achieved by T-DNAs or transposons (Krysan et al., 1999; Parinov and Sundaresan, 2000). The simplicity of the technique combined with the high gene density in the *Arabidopsis* genome (Bevan et al., 2001) has led to the creation of several insertional mutant populations (Krysan et al., 1999; Maes et al., 1999; Walbot, 2000; Ramachandran and Sundaresan, 2001; Emmanuel and Levy, 2002; May and Martienssen, 2003). Nevertheless, loss-of-function mutants have faced limitations, e.g., due to functional gene redundancy caused by extensive genome

duplications present in *Arabidopsis* (*Arabidopsis* Genome Initiative, 2000; Bouche and Bouchez, 2001). Thus, alternative approaches needed to be developed to overcome this drawback. One of them is the generation of gain-of-function mutagenesis, in which a gene is either ectopically or constitutively overexpressed. Although in such a case a particular gene is not expressed in its normal biological context, it may provide information about biological processes, in which the gene plays an important role.

Activation tagging

Activation tagging is a gain-of-function mutagenesis approach and it was first described to identify and isolate novel genes from tobacco (Walden et al., 1994), although it had been previously reported with *in vitro* experiments to activate auxin-related plant genes (Hayashi et al., 1992). Since then, it has been extensively improved by the establishment of large collections of *Arabidopsis* lines (Kardailsky et al., 1999; Borevitz et al., 2000; van der Graaff et al., 2000; Weigel et al., 2000; Marsch-Martinez et al., 2002; Nakazawa et al., 2003). The system used for these collections are based on quadruple *CaMV35S* enhancer sequences as activator elements, either with T-DNA or transposon as insertional vehicles (Weigel et al., 2000; Marsch-Martinez et al., 2002). The frequency of morphological mutants varies depending on the insertion vehicle used, with approximately 0.1% in the case of the T-DNA-based system, or about 1% when transposons are used. This low frequency in the T-DNA based activation tagging is mainly attributed to the fact that the 4x*35S* enhancer sequences are methylated (Chalfun-Junior et al., 2003). Although very successful, activation tagging possesses disadvantages, such as that the observed phenotype may not be directly associated with the function of the affected gene. Nevertheless, new populations are being generated not only in *Arabidopsis*, but also in other species such as tomato (Mathews et al., 2003), rice (Jeong et al., 2002), petunia (Zubko et al., 2002), and tobacco (Ahad et al., 2003), demonstrating the strength of the system.

Table 1. List of useful *Arabidopsis* websites[1]

Name	Short description	URL
ABRC	*Arabidopsis* Biological Resource Center. Plant resources	http://arabidopsis.org/abrc/
AFFYMETRIX	*Arabidopsis* Genome Array	http://www.affymetrix.com/
AFGC	NSF-funded DNA microarray analysis and T-DNA Knockout facilities	http://arabidopsis.org/info/2010_projects/comp_proj/AFGC/index.html
AGR	Provides information for identifying gene function and crop plant orthologues of *Arabidopsis* genes	http://flora.life.nottingham.ac.uk/agr/
AGRIS	The website is an information resource of *Arabidopsis* promoter sequences, transcription factors, and their target genes	http://arabidopsis.med.ohio-state.edu/
ARABINET	*Arabidopsis* links	http://weeds.mgh.harvard.edu/atlinks.html
ARAMENON	A specialized database for *Arabidopsis* thaliana membrane proteins	http://aramemnon.botanik.uni-koeln.de/
ATDB	*Arabidopsis* insertion database	http://www.atidb.org/
AthaMap	Genome-wide map of putative transcription factor binding sites in *Arabidopsis*	http://www.athamap.de/
ATIS	Links to Transposon and T-DNA resources for insertional mutagenesis, gene traps, promotor traps, enhancer traps, and activation tags based on transposons and T-DNAs	http://www.jicgenomelab.co.uk/atis/
AtRepBase	Database of genomic sequences repeated in the *Arabidopsis* genome	http://nucleus.cshl.org/protarab/AtRepBase.htm
Biological Resource Center (Hungary)	Search for insertions by chromosome, locus name, At number or protein function	http://www.szbk.u-szeged.hu/~arabidop/
BIOSCI	*Arabidopsis* newsgroup	http://www.bio.net/hypermail/ARABIDOPSIS/
CATMA	Complete *Arabidopsis* transcriptome microarray: gene sequence tags	http://www.catma.org
CSHL	CSHL Gene trap and Enhancer trap transposon lines. Search the database by BLAST or keyword.	http://genetrap.cshl.org/
FST (INRA - Versailles)	Search the Flanking Sequence Tags database by online BLAST with your gene sequence	http://flagdb-genoplante-info.infobiogen.fr/projects/fst/DocsIntro/Page_accueil.html
GABI-Kat	T-DNA insertion database	http://www.mpiz-koeln.mpg.de/GABI-Kat/
GARNET	UK *Arabidopsis* functional genomics network	http://www.york.ac.uk/res/garnet/garnet.htm

Table 1. Continued…

Name	Short description	URL
KAZUSA	Database *Arabidopsis* genome sequencing and re-annotation and cDNA analysis	http://www.kazusa.or.jp/en/plant/
KOAS	The KAZUSA *Arabidopsis* opening site	http://www.kazusa.or.jp/kaos/
Lehle Seeds	Private company selling *Arabidopsis* seeds and growing systems	http://www.arabidopsis.com/
MASC SNP DB	SNP database at MPIZ Köln	http://www.mpiz-koeln.mpg.de/masc/index.html
MIPS	MIPS *Arabidopsis* genome database	http://mips.gsf.de/proj/thal/proj/thal_overview.html
Mutants genes of *Arabidopsis*	This page provides links to detailed information on rules of nomenclature, mutant gene symbols, linkage data, genetic maps, and e-mail addresses of contributing laboratories	http://mutant.lse.okstate.edu/genepage/genepage.html
NASC	Provides seeds outside of America	http://nasc.nott.ac.uk/
RARGE (Riken *Arabidopsis* + Genome Encyclopedia	RARGE is a web site that provides services for resource data searching. It presents *Arabidopsis* resource data (cDNAs, tranposon mutants and microarray experiments) for all biologic researchers.	http://rarge.gsc.riken.go.jp/index.html
Rfam UK	micro-RNA database for micro-RNA sequences from *A. thaliana* and other species	http://www.sanger.ac.uk/Software/Rfam/mirna/
RIKEN (BioResource Centre)	Center for distributing plant seeds, plant cDNA and plant cell culture	http://www.brc.riken.go.jp/lab/epd/Eng/index.html
RIKEN (functional genomics)	Plant functional genomics research group in Japan	http://pfgweb.gsc.riken.go.jp/index.html
SASSC	The SENDAI *Arabidopsis* Seed Stock Center. Maintains over a 1100 unique lines of *Arabidopsis* and related species	http://www.shigen.nig.ac.jp/arabidopsis/
SeedGenes	Genes essential for *Arabidopsis* development	http://www.seedgenes.org
SIGnAL	Database for T-DNA insertions. Search by name, function, or online BLAST to find insertions in your gene of interest	http://signal.salk.edu/cgi-bin/tdnaexpress
TAIR	The *Arabidopsis* Resource Information	http://arabidopsis.org
TIGR	The TIGR *Arabidopsis* database	http://www.tigr.org/tdb/e2k1/ath1/
TILLING	*Arabidopsis* Tilling Project	http://tilling.fhcrc.org:9366/

[1]/ Most of the websites listed were obtained from the TAIR website.

Aim and outline of this thesis

The general aim of this thesis was to study the molecular control of plant development, especially plant architecture, using novel mutants obtained by a transposon-based activation tagging approach in *Arabidopsis thaliana*. The obtained mutants were selected on the basis of aberrations in the flower.

Chapter 1 briefly introduces the world of plant architecture and *Arabidopsis* research and the main aspects dealt within this thesis. **Chapter 2** reviews how activation and suppression control plant development at various stages of the plant life cycle. Examples are given how developmental switches are regulated. **Chapter 3** deals with tissue specific activation tagging demonstrating that the use of specific enhancer elements enables ectopic expression of genes. **Chapter 4** covers one of the drawbacks of the T-DNA based activation tagging in *Arabidopsis*: the low frequency of mutants. This chapter shows that there is a correlation between the low efficiency of the approach and silencing of the 4x35 S enhancer sequences due to methylation. In **Chapter 5**, the molecular analysis of a transposon-based activation-tagging mutant called *downwards siliques1* (*ds1-D*) is described. This study reveals that *DS1* is important to control the blade-sheath boundary in the *Arabidopsis* petals. In **Chapter 6**, another gain-of-function mutant, *needle1-D* (*ndl1-D*) is described. The studies performed with this mutant suggest that the *NDL1* gene controls carpel development in *Arabidopsis* and most likely acts in auxin transport. **Chapter 7** exhibits the results obtained by the analysis of *TWISTED1* (*TWT1*), which was identified by the third gain-of-function mutant, *twisted1-D*. Overexpression of *TWT1* affects plant architecture and moreover, the results of the overexpression phenotype combined with the expression analysis suggest that the *TWT1* gene is involved in vascular tissue development. In **Chapter 8** the results and perspectives of studies on plant architecture using activation tagging are summarized.

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CHAPTER 2

Introduction

Development is a core topic in plant research. The way in which a single cell becomes a complex organism has intrigued scientists all over the world for many centuries, and they have used many approaches to reach understanding of this major enigma of the plant life cycle. What are the factors that control the behaviour of cells towards division, elongation, differentiation and death? These "simple" steps in the life of a cell are the basis for the further development of the organism and the formation of tissues and organs. These cellular activities are not restricted to any phase of the plant's life cycle, but rather, follow a continuous programme required to produce new structures such as roots, leaves, flowers and gametes, which ensure the formation of the next generation. To understand plant development it is necessary to analyse the cellular behaviour at various stages of development and to unravel the molecular genetic mechanisms underlying these processes. These mechanisms are complex networks of factors that interact with each other and control processes by activation and suppression. By the action of antagonists and agonists ‘switches’ are generated that can either initiate or shut off biological processes in the cell that lead to development and growth. A perfect model species to investigate these biological switches is *Arabidopsis thaliana*, which has become the worldwide used plant species for developmental studies. This is particularly stimulated by the availability of the complete genome sequence (*Arabidopsis* Genome Initiative, 2000) and the extensive databases and plant resources. In this chapter, we give an overview of regulatory mechanisms active throughout *Arabidopsis* development with special emphasis on activation and suppression control.

The approaches to use

Researchers have used a variety of approaches to reach the goal of understanding the molecular processes of plant development. These approaches are mainly concentrated on the identification of gene functions, which is the basis for unravelling important regulatory networks. Two fundamental tools have been used for this purpose: forward and reverse genetics. Forward genetics is based on a random mutagenesis approach and selecting the desired phenotype, followed by the identification and cloning of the gene responsible for the altered phenotype. In contrast, reverse genetics begins with a gene of interest and subsequently, generation of the corresponding mutant line. After the sequence completion of the *Arabidopsis* genome, reverse genetics has become more used. A variety of strategies have been devised to generate and isolate mutants from known genes of *Arabidopsis* and other plant species by T-DNA or transposon insertional mutagenesis (Krysan et al., 1999; Maes et al., 1999; Walbot, 2000; Ramachandran and Sundaresan,

2001; Emmanuel and Levy, 2002; May and Martienssen, 2003). The use of these approaches is attributed mainly to the convenience to generate large populations in which reverse screenings can be performed in a high throughput manner. Nowadays, the flanking sequences of the insert (either T-DNA or transposon) are known, allowing searching in databases for lines with an insert in almost any gene of interest. Examples of these publicly available databases are: SALK (http://signal.salk.edu/cgi-bin/tdnaexpress), GABI_Kat (http://www.mpiz-koeln.mpg.de/GABI-Kat/) and ATIDB (http://www.atidb.org/). These mutant resources together reach a near to saturation situation, with more than 225.000 independent T-DNA insertion events in the *Arabidopsis* genome (Alonso et al., 2003), resulting in the identification of mutations in more than 21.700 of the 29.454 predicted genes.

Although many *Arabidopsis* mutants have been obtained by insertional mutagenesis approaches, only a few have an altered phenotype that is informative about the gene function (Bouche and Bouchez, 2001). An explanation for this limitation is gene redundancy, a phenomenon that appears to be very common in *Arabidopsis* (*Arabidopsis* Genome Initiative, 2000). It was estimated that approximately 40% of the genome is duplicated and that the copies still retain partial (or complete) overlapping functions. To partially overcome this problem RNA interference (RNAi) strategies, that enable the down-regulation of homologous genes in parallel are being used (Chuang and Meyerowitz, 2000). Although RNAi can be very effective, it does not lead to complete knockouts and multiple genes can be affected, even when that is unwanted.

Alternative methods have been created to overcome the drawbacks of the loss-of-function reverse genetics strategies in plants. Overexpression of a gene using a constitutive promoter offers an alternative and complementary strategy to knockout analysis, especially because, sometimes, gene functions are revealed only when the gene is overexpressed (van der Graaff et al., 2000; Pontier et al., 2001; Fan and Dong, 2002). Moreover, activation tagging (Weigel et al., 2000; Marsch-Martinez et al., 2002) has become a useful tool to generate gain-of-function dominant mutations. This approach is based on a strong enhancer such as that derived from the Cauliflower Mosaic Virus (*CaMV*) *35S* promoter. Several reported examples have shown the power of this technique (Hayashi et al., 1992; Kardailsky et al., 1999; van der Graaff et al., 2000; Marsch-Martinez et al., 2002; Mathews et al., 2003; Nakazawa et al., 2003; Yuen et al., 2003). The activation tagging approach based on transposable elements, which was recently reported by Marsch-Martinez et al. (2002), is particularly attractive because it does not lead to silencing of the enhancers, a phenomenon that appears to be more common for the T-DNA based activation tagging (Chalfun-Junior et al., 2003). Activation tagging has also been used to isolate suppressor mutants of already known loss-off-function mutant phenotypes (Neff et al., 1999; van der

Graaff et al., 2003). Therefore, activation tagging, may lead to opposite phenotypes compared to loss-of-function mutants.

Even when a loss-of-function mutant gives a phenotype, overexpression can still be informative because it may generate a completely unforeseen phenotype (Wada et al., 1997), which helps in the identification of gene function. A loss-of-function mutant reflects the unique contribution of a gene to a specific function, while overexpression is often associated with broader changes in phenotype that can be divided into two types: hypermorphs (high forms) and neomorphs (new forms) (Zhang, 2003). Hypermorphs are overexpressors in which the introduced gene confers the same function as the endogenous gene but with higher activity. In neomorphs, the introduced protein (because of its abundance or inappropriate tissue and/or developmental stage context) confers a new function that is not present in the wild type. Although neomorphs might be interesting for biotechnological use, they are of limited value for gene function determination. Therefore, overexpression data should be interpreted with care and preferably combined with loss-off-function mutant information.

In addition to the above described forward and reverse mutagenesis strategies to elucidate gene functions, the use of natural variation, as a forward genetics approach is becoming more important (Alonso-Blanco and Koornneef, 2000).

In conclusion, *Arabidopsis* and the research community around this model species offer an extensive toolbox for gene function analysis. The challenge will be to integrate these gene function data, which should lead to the understanding of genetic networks controlling processes in the plant life cycle.

Activation and suppression during the plant life cycle

Like other Angiosperms, *Arabidopsis* starts its life cycle with the embryogenesis programme, followed by germination and elongation of shoots and roots. During embryogenesis the body patterns are established and the root and shoot apical meristems are formed. A major developmental switch is the phase change from vegetative to reproductive growth, after which the plant enters the flowering phase. New types of meristems are formed, e.g. inflorescence and floral meristems. During the reproductive phase, the gametes are formed, which merge during the fertilisation process. Fertilisation brings together the genetic information from the male and from the female side, but also additional information can be inherited from either of the partners, a process that is known as maternal/paternal imprinting. All these major developmental phases are controlled at the cellular level but requires intercellular communication to establish the right patterns and organs. In addition to the internal regulatory circuits, plant architecture is also influenced by environmental conditions, such as light, temperature, humidity, and nutrient status. For these external factors, receptors are required to perceive the signal and transfer it to a

signal transduction system within the cell. Finally, this will lead to transcription of genes that cause a response of the cell and the organism. A major class of regulators of plant development are the transcription factors. It has been predicted that the *Arabidopsis* genome contains approximately 1500 transcription factors. Because these transcription factors can interact with each other at the protein level, an enormous regulatory network is formed, which is dynamic during plant development.

It is known that transcription factors can act as activators or suppressors, and consequently their regulation is a key point in plant development. Another type of transcription regulators are the chromatin remodelling proteins. They determine the status of the chromatin and the availability of the DNA for transcription initiation. In general, these chromatin remodelling proteins suppress the transcription of genes, in particular during major developmental switches (Reyes et al., 2002). Developmental switches are only possible when the previous stage is suppressed and the new phase is initiated. Therefore, suppression and activation are two interlinked processes.

Below are examples given of major regulators of different developmental programmes, with an emphasis of antagonistic action of these regulatory factors.

Embryogenesis

LEAFY COTYLEDON1 (*LEC1*) is a central regulator during the early and late phases of embryogenesis. In *lec1* mutants the embryonic identity is lost before germination, resulting in embryonic cotelydons with leaf-like properties. Overexpression of *LEC1 (35S::LEC1)* yielded plants exhibiting somatic embryo formation on vegetative tissues of the plant (Kwong et al., 2003). A similar phenotype was obtained when the AP2-like transcription factor *BABYBOOM (BBM)* was overexpressed (Boutilier et al., 2002). These mutants indicate that *LEC1* and *BBM* are required to determine or maintain embryonic identity. At germination the embryonic identity is repressed and vegetative identity appears, a phase change in which the *PICKLE (PKL)* gene plays a major role. *PKL* encodes a chromatin remodelling factor, which is necessary for the repression of *LEC1* (Dean Rider et al., 2003). In *pkl* mutants, levels of *LEC1* and other important genes involved in embryogenesis are upregulated more than 100 fold. Consistently with the expression results, *pkl* mutant seedlings are unable to repress embryonic identity (Dean Rider et al., 2003), suggesting that *PKL* has a suppression mode of action on embryogenesis genes such as *LEC1* and *BBM.*

Shoot apical meristem (SAM) formation

In plants, two populations of cells, forming the shoot apical meristem (SAM) and the root apical meristem (RAM), respectively, together are responsible for the formation of the entire adult plant. The meristems are established early in embryogenesis and maintained throughout the plant life. The SAM is located at the extreme tip of the shoot, as a group of undifferentiated and highly organised cells (stem cells) that continuously produce lateral organs (leaves and flowers) (Takada and Tasaka, 2002; Weigel and Jürgens, 2002; Byrne et al., 2003).

Two processes are of major importance within a plant meristem: (i) the control of stem cell proliferation and (ii) the balance between maintenance of stem cell identity and differentiation. For controlling proliferation and meristem size, two factors regulate each other by a negative feedback mechanism. The homeobox gene *WUSCHEL* (*WUS*) is required for stem cell maintenance and meristem expansion. In contrast, the *CLAVATA* complex, composed of the ligand *CLV3* and the receptor kinase dimer *CLV1/CLV2* is responsible for the suppression of cell proliferation in the meristems (Schoof et al., 2000). *wus* mutant seedlings are arrested in growth due to a loss of meristematic cells (Laux et al., 1996), while *clv* mutants show the opposite, i.e. accumulation of undifferentiated cells and an enlargement of the meristems (Clark et al., 1993; Clark et al., 1995; Kayes and Clark, 1998). The way the *clv* mutants accumulates undifferentiated cells in the shoot and floral meristems suggests an interaction with *WUS*. *WUS* expression in the wild type is restricted to a small number of cells located centrally in the lower cells of the L3 layer of the shoot meristem (Mayer et al., 1998; Schoof et al., 2000), whereas in *clv* mutants *WUS* expression expands both apically and laterally (Schoof et al., 2000). Moreover, ectopic expression of *WUS* under the control of the *CLV1* promoter is sufficient to accumulate stem cells at the shoot (Schoof et al., 2000), exactly as in *clv* mutants. These findings indicate that the *clv* phenotype is a result of *WUS* misexpression, demonstrating the antagonistic regulation of meristem size by WUS and CLV proteins.

Another important group of genes involved in the maintenance of meristematic activity are the class I type of the *KNOTTED-LIKE* genes or *KNOX* genes (Reiser et al., 2000). This class comprises e.g., *SHOOT MERISTEMLESS* (*STM*), *KNAT1* (also known as *BREVIPEDICELLUS* - *BP*), *KNAT2* and *KNAT6*. Importantly, most of the identified class I genes are expressed throughout the meristem but not in lateral organs (Reiser et al., 2000). Their loss-of-function mutations show that they are involved in the determination of cell fate and meristem patterning (Barton and Poethig, 1993; Endrizzi et al., 1996; Long et al., 1996). *stm* mutant seedlings display fused cotelydons, lack a SAM and do not develop post-embryonic vegetative tissues (Barton and Poethig, 1993; Endrizzi et al., 1996). In contrast, a mutation in the *Arabidopsis BP* gene alone does not cause meristem defects (Lincoln et al., 1994; Byrne et al., 2002), but instead, it leads to a change in plant

architecture, such as organs bent downwards and shorter internodes (Douglas et al., 2002; Venglat et al., 2002). *STM* regulates organ initiation in the SAM by repressing two genes that promote organ formation, *ASYMMETRIC LEAVES1* (*AS1*) and *ASYMMETRIC LEAVES2* (*AS2*), which in turn negatively regulate the expression of *KNOX* genes (Byrne et al., 2000; Ori et al., 2000; Semiarti et al., 2001; Byrne et al., 2002). Suppression of *KNOX* genes in lateral organ primordia is required for differentiation and organ patterning. Thus, downregulation of *STM* allows the expression of *AS1* and *AS2* in organ primordia, which in turn repress meristematic cell fate by suppressing genes such as *KNAT1*, *KNAT2*, *KNAT6* (Byrne et al., 2000; Ori et al., 2000; Semiarti et al., 2001; Byrne et al., 2002). In line with this, the overexpression of *KNOX* genes in different plant species resulted in altered leaf morphology and the formation of ectopic meristems (Lincoln et al., 1994; Reiser et al., 2000). Such a phenotype is similar to mutations in the *AS1* and *AS2* genes, demonstrating that the *AS2* and *KNOX* genes have antagonistic effects. When *AS2* is overexpressed by the *35S* promoter a phenotype is obtained similar to the *bp* loss-of-function mutant with a repression of other *KNOX* genes (Lin et al., 2003; Nakazawa et al., 2003). Similar defects are apparent when the closest homolog of *AS2*, *DOWNWARDS SILIQUES1* (*DS1*), is upregulated by the *35S* promoter (this thesis). *AS2* and *DS1* belong to the recently identified *LATERAL ORGAN BOUNDARY* (*LOB*) family (Shuai et al., 2002). Based on the functional analysis of a few members of the LOB family, it seems that these genes play a role in lateral organ differentiation by excluding expression of *KNOX* genes outside the shoot meristem, establishing a boundary between organ tissues and meristems.

Plant hair formation

Trichomes, as well as roots hairs, are hair-like structures that are formed from a single highly specialised epidermal cell. This cell undergoes differentiation and growth, and a number of mutants affected in trichome development have been reported (Marks, 1997). Genetic analyses of trichome and root hair development revealed a remarkable similarity between the regulation of these two types of plant hairs. Two genes, *GLABRA1* (*GL1*) and *TRANSPARENT TESTA GLABRA1* (*TTG1*) play important roles in trichome development (Koornneef et al., 1982). Mutations in *GL1* affects only the trichomes of the shoot epidermis (Koornneef et al., 1982), whereas *ttg1* mutant plants exhibit further aberrations, such as increased numbers of root hairs and anthocyanin defects (Galway et al., 1994). Another recessive mutant, *gl2* is also affected in trichome morphogenesis and induces ectopic root hairs (Koornneef et al., 1982). In contrast to *ttg1* and *gl2*, *caprice* (*cpc*) mutants fail to produce root hairs (Wada et al., 1997). Overexpression of *CPC* by the *35S* promoter shows ectopic hairs in roots, exactly as in *ttg1* and *gl2* mutant plants (Wada et al., 2002). In addition to that, *35S::CPC* transgenic plants miss trichomes on leaves, stems and sepals (Wada et al., 2002), suggesting that the regulation of trichomes and root

hairs, are partially controlled by common pathways. Moreover, based on the results of *CPC* overexpression and the mutant phenotypes, it was demonstrated that *TTG1* and *GL2* act antagonistically with *CPC* in regulating root hair and trichome formation (Wada et al., 2002).

Flowering

Timing of the transition from the vegetative to the reproductive phase, flowering time, is regulated by a set of genes acting either in the same or in independent pathways (Blazquez, 2000). This complex genetic network is composed of suppressors and activators of flowering which perceive both external and internal signals that are transduced to the central integrator, *LEAFY* (*LFY*). *LFY* belongs to the meristem-identity genes, which determine the identity of the floral meristem. In *Arabidopsis* it is expressed in the floral primordia just after the switch from vegetative to reproductive growth (Weigel et al., 1992). Another meristem identity factor, *APETALA1* (*AP1*) acts together with *LFY* during this transition (Parcy et al., 1998). Overexpression of *LFY* and *AP1* under the control of the *CaMV35S* promoter resulted in much earlier formation of flowers and a determinate inflorescence (Mandel and Yanofsky, 1995). This early flowering phenotype resembles the phenotype of mutant plants affected in another meristem identity gene, *TERMINAL FLOWER* (*TFL1*) (Liljegren et al., 1999). *TFL* acts antagonistically to *LFY* and *AP1* and is required for the maintenance of the inflorescence meristem and suppressing floral meristem identity. In line with this function, overexpression of *TFL* leads to late flowering plants in which the floral meristems are replaced by inflorescence structures, reminiscent to the double *lfy ap1* mutant phenotype (Ratcliffe et al., 1998).

After initiation of floral meristems, the floral homeotic genes determine the identity of the floral organs. The action of these identity genes has been described in the well-known ABC model (Bowman et al., 1991; Coen and Meyerowitz, 1991). Class A encompasses the genes *APETALA1* (*AP1*) and *APETALA2* (*AP2*); class B, the genes *APETALA3* (*AP3*) and *PISTALLATA* (*PI*); and class C function is represented by *AGAMOUS* (*AG*). These genes act alone or in combination to determine the identity of floral organs in an overlapping manner. Expression of class A genes alone induces sepal formation, whereas the expression of A and B genes leads to petals, B and C class genes expression results in stamen identity and when the C gene is expressed alone, it confers carpel identity (Bowman et al., 1991; Coen and Meyerowitz, 1991). The first ABC gene isolated was *AG* (Yanofsky et al., 1990). *ag* mutant plants produce flowers in an indeterminate pattern: (sepals, petals, petals)$_n$ (Bowman et al., 1991), whereas the overexpression of *AG* using the *35S* promoter leads to carpel formation in stead of the sepals and stamens replacing the petals in the second whorl. Interestingly, this phenotype shows similarity to the *ap2* mutant in which the first whorl is also homeotically

transformed into carpels (Mizukami and Ma, 1992). This indicates that *AG* is spatially controlled by the class A gene *AP2* and *vice versa*, AP2 activity is suppressed by *AG*. Regulatory networks controlling organ fate are also extended to fruit morphogenesis. The *FRUITFULL* (*FUL*) gene is necessary for valve development in gynoecium and silique formation (Gu et al., 1998). *ful* mutant plants exhibit siliques that fail to elongate after fertilisation, caused by the lack of valve expansion. In contrast, the replum and septum cells continue to elongate (Gu et al., 1998). Mature *ful* siliques fail to dehisce normally, because of the abnormal valve-replum boundary formation. When *FUL* is overexpressed by the *35S* promoter, it shows a replum-to-valve conversion, demonstrating that *FUL* specifies valve identity. The replum, which contains the dehiscence zone, is specified by two redundant genes, *SHATTERPROOF1* (*SHP1*) and *SHATTERPROOF2* (*SHP2*) (Liljegren et al., 2000). Mutation in these genes affects the differentiation of the dehiscence zone and abolishes lignification of cells and the subsequent dispersal of the seeds from dried siliques ("shattering") (Liljegren et al., 2000). Genetic analysis showed that *SHP* and *FUL* genes interact antagonistically. Firstly, the phenotype of *shp1shp2* double mutant resembles the *35S::FUL* gain-of-function phenotype (Gu et al., 1998; Ferrandiz et al., 2000; Liljegren et al., 2000). Secondly, like *35S::SHP 35S::SHP2* siliques, *ful* siliques exhibit, among other defects, lack of lateral valve expansion, ectopic lignification and seed crowding (Gu et al., 1998; Ferrandiz et al., 2000; Liljegren et al., 2000). In conclusion, *FUL* negatively regulates *SHP* genes and *vice versa* in order to specify a boundary between valve margin cells and the replum cells.

An antagonistic interaction mechanism is also active during carpel development. Overexpression of *NEEDLE1* (*NDL1*) (this thesis), shows a conspicuous phenotype similar to loss-of-function mutations in genes involved in carpel development and formation, such as *ETTIN* (*ETT*) (Sessions and Zambryski, 1995; Sessions et al., 1997; Heisler et al., 2001) and *PINOID* (*PID*) (Christensen et al., 2000; Benjamins et al., 2001). In these mutants, carpel formation is disrupted, most likely due to defects in auxin transport.

Another gain-of-function mutant, *TWISTED1* (*TWT1*) also displays an aberrant fruit phenotype (this thesis). The silique of this mutant is twisted, resembling recessive mutants that affect cell expansion patterns, such as in *lopped1* (*lop1*) (Carland and McHale, 1996) or *tornado1-1* (*trn1-1*) mutants (Cnops et al., 2000). Both loss-of-function mutants exhibit twisted siliques, apart from other defects in plant morphology.

Conclusions and perspectives

The formation of an organism is an extremely complex process, which has to be tightly regulated and fine-tuned at all levels. Cells undergo differentiation and acquire a certain identity. Furthermore, the direction and timing of differentiation and timing of cell growth are essential for the morphogenesis of tissues and organs. Obviously, most of these

processes are genetically determined and include genes that either promote or suppress pathways. In this review, we discussed examples showing that these regulatory mechanisms are important at various stages of plant development (Figure 1). Nowadays, a number of *Arabidopsis* factors are known that act antagonistically and function as biological switches. As switches they control the transitions to other phases, e.g. from vegetative to reproductive phase, or they play a role in establishing boundaries between meristematic and differentiated cells or between cell types with different identities. These switches are central in molecular networks, but also facilitate the connection points between the networks controlling developmental programmes. The challenge for the future is to further unravel these complex networks and the components involved. The results described in this thesis demonstrate that activation tagging is a powerful tool that could substantially contribute to our understanding of the regulatory network underlying plant development.

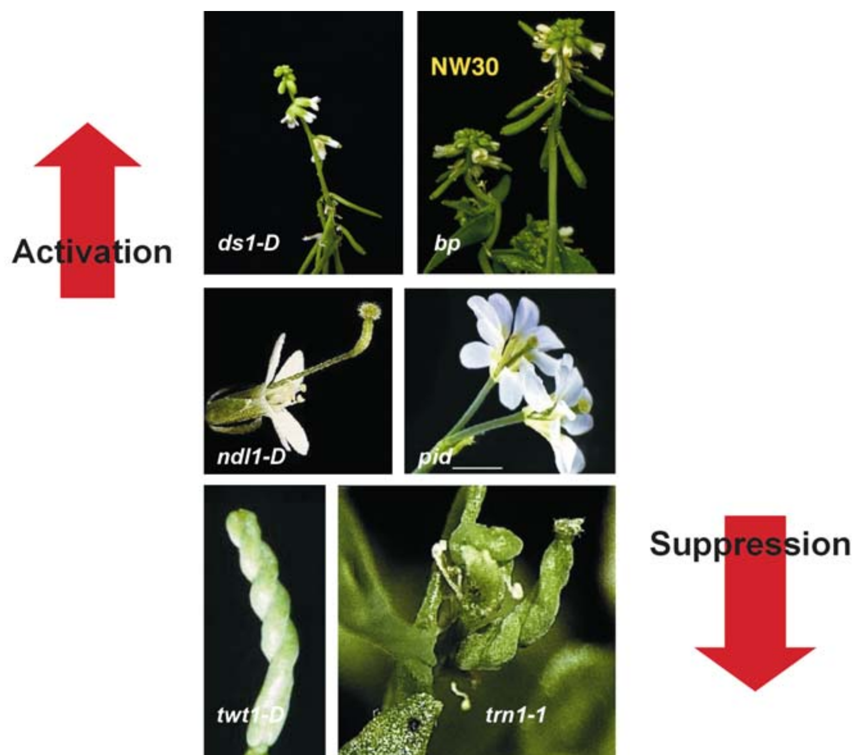

Figure 1. Examples of activation and suppression during various stages of plant development. Gain-of-function mutants (*ds1-D*, *ndl1-D* and *twt1-D*) resemble loss-of-function mutants (*bp*, *pid* and *trn1-1*), demonstrating that biological regulatory switches act antagonistically in molecular networks controlling developmental programmes. *bp* picture was obtained from the Nottingham *Arabidopsis* Stock Centre (NASC, http://nasc.nott.ac.uk/) and the *pid* picture was from Benjamins et al. (2001).

Acknowledgements

Vagner Augusto Benedito is acknowledged for ideas and discussion on the review and also Ruud de Maagd for carefully reading the manuscript. The author Antonio Chalfun-Junior has been financially supported by CAPES-Brazil (BEX 1519/98-0).

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CHAPTER 3

Analysis of the *SHP2* enhancer for the use of tissue specific activation tagging in *Arabidopsis thaliana*

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Abstract

Activation tagging is a powerful tool to identify new mutants and obtain information about possible biological functions of the overexpressed genes. The quadruple cauliflower mosaic virus (*CaMV*) *35S* enhancer fragment is a strong enhancer, which is most commonly used for this purpose. However, the constitutive nature of this enhancer may generate lethal mutations or aberrations in different plant organs by the same overexpressed gene. A tissue-specific activation tagging approach may overcome these drawbacks and may also lead more efficiently to the desired phenotype. For this reason the *SHATTERPROOF2* (*SHP2*) promoter fragment was analysed for enhancer activity. The *SHP2* gene is involved in dehiscence zone development and expressed during silique development. The aim of the experiments described here was to identify a dehiscence zone specific enhancer that could be used for tissue specific activation tagging. The chosen *SHP2* enhancer fragment was found to be expressed predominantly in the dehiscence zone and showed enhancer activity as well as ectopic expression activity. This activity was not influenced by its orientation towards the promoter and it was still functional at the largest tested distance of 2.0 kb. Based on these results, the *SHP2* enhancer fragment can potentially be used in a tissue-specific activation tagging approach to identify new *Arabidopsis* mutants with an altered dehiscence zone formation.

Introduction

Activation tagging has become an upcoming tool to generate mutant plant. It is an alternative approach for gene function analysis, because loss-of-function mutations has its limitation in cases of functional gene redundancy (*Arabidopsis* Genome Initiative, 2000). Activation tagging was proposed as a novel gene isolation method in which a gene is either ectopically or constitutively overexpressed compared to normal expression levels (Walden et al., 1994). Walden et al. (1994) designed a T-DNA based activation tagging approach to identify and isolate novel genes from tobacco and since then, it has been largely applied using either T-DNA insertion strategies (Borevitz et al., 2000; Ito and Meyerowitz, 2000; Lee et al., 2000; van der Graaff et al., 2000; Weigel et al., 2000; Huang et al., 2001) or transposon based approaches (Wilson et al., 1996; Marsch-Martinez et al., 2002). This technology has been applied successfully to many plant species like *Arabidopsis*, rice, tomato, petunia and tobacco (Weigel et al., 2000; Jeong et al., 2002; Zubko et al., 2002; Ahad et al., 2003; Mathews et al., 2003).

Activation tagging is based on strong transcriptional enhancer sequences that can activate gene expression in the vicinity of the site where the enhancer was inserted into the genome. The most commonly used enhancer is a quadruple combination of the cauliflower mosaic virus (*CaMV*) *35S* enhancer (Odell et al., 1985; Hayashi et al., 1992). These 4x*35S* enhancer elements have been reported to strongly enhance endogenous gene expression rather than ectopically or constitutively overexpress genes (Neff et al., 1999; van der Graaff et al., 2000). It has been demonstrated that it can stimulate gene expression of neighboring genes independently of its orientation, up to a distance of 3.6 kb (Weigel et al., 2000) or even up to 5.0 kb (this thesis).

It is tempting to use this technique in a random like approach to isolate new mutants and analyse in more detail the overexpressed genes. In some cases the mutant displays a phenotype that can either be directly associated with the gene function of the activated gene (Zubko et al., 2002) or may provide an indication of the pathway in which the gene is involved (Kardailsky et al., 1999; Zhao et al., 2001; Yuen et al., 2003). The activation tagging method has also been used as a novel approach to isolate suppressor mutants of known mutant phenotypes (Neff et al., 1999; van der Graaff et al., 2003). However, when searching for specific mutants with a more specific phenotype, the 4x*35S* enhancer is not very attractive to use, as it will induce aberrations in more plant tissues than the specific one you would like to modify. In that case, tissue specific enhancement could directly lead to the mutants of interest. To test this hypothesis we have characterized a tissue specific enhancer that may be applicable to efficiently generate activation mutants with an altered pod shattering phenotype.

Precocious pod shattering is a phenomenon that fruits lose their seeds before harvesting time, generating high losses of agronomic crops, e.g., rapeseed. Engineering for

shatter resistant plants allow the plants to grow until the fruits are fully ripened, resulting in an optimal product quality and increased crop yield. The closely related *MADS* box transcription factors *SHATTERPROOF1* (*SHP1*) and *SHATTERPROOF2* (*SHP2*) control seed dispersal in *Arabidopsis* by regulating the development of the dehiscence zone (Ferrandiz et al., 2000; Liljegren et al., 2000; Ferrandiz, 2002). In *shp1shp2* double mutant plants, the dehiscence zone is completely absent and the fruits fail to open.
In this chapter, we describe the identification of a *SHP2* enhancer taken from its natural promoter, which contains all necessary characteristics required for a tissue specific activation approach.

Material and methods

Plant material and transformation

Constructs were introduced into *Arabidospis thaliana* ecotype Col-0 using *Agrobacterium tumenfaciens* strain GV3101 and the floral dip method (Clough and Bent, 1998). For selection of transformants, seeds were surface sterilized by vapor phase sterilization and selected on medium containing kanamycin. Plants were grown in soil, under normal greenhouse conditions (22 ºC, 14/10 hours light/dark).
Constructs

The *SHP2* enhancer fragment

Primers were designed at –1275 bp of the transcription initiation site of the *SHP2* gene (forward primer *SHP2*F 5’-AAGTTCTTTCTTGAAATG-3’) and –55 bp situated from the same transcription initiation site (reverse primer *SHP2*R 5’-CACTTAACTGCTGCTTCAAC-3’). Both primers were extended with sequences for digestion sites to facilitate subcloning. DNA of *Arabidopsis thaliana* ecotype Col-0 was used as template for PCR amplification. Using these primers a band of the expected 1220 bp was obtained, cloned (in pGEMT-Easy®, Promega) and sequenced to confirm its identity. We will refer to this sequence as the *SHP2* enhancer fragment.

SHP2 enhancer fused to minimal *–47-35S::GUS*

The minimal *-47-35S* promoter *GUS* vector, *GUS*XX-47 (Pasquali et al., 1994) was obtained from J. Memelink, (Leiden University, Leiden, The Netherlands). The *SHP2*F primer was extended with a *Hin*dIII site and the reverse primer with a *Sal*I site, the fragment amplified and ligated in the *Hin*dII–*Sal*I site upstream of the *-47-35S* promoter, which was fused to the *GUS* reporter gene. The whole fragment was subcloned in pBINPLUS (van Engelen et al., 1995) using *Hin*dIII-*Kpn*I, resulting in vector pGD751.

SHP2* fragment fused to the *pFBP1::GUS

Both *SHP2* primers were extended with both *Hin*dIII and *Sal*I site to facilitate the cloning of the vectors described next. Several constructs were generated to test the enhancer activity of the *SHP2* fragment. For that reason the *SHP2* fragment (as a *Hin*dIII-*Hin*dIII fragment) was ligated upstream the *FBP1* promoter present in vector pFBP12E (Angenent et al., 1993). This resulted in a sense fusion of the *SHP2* enhancer to the 'short' 220 bp *pFBP1::GUS* (pGD418).

The *Sal*I-*Sal*I fragment was introduced in the *Sal*I digested pFBP12E vector, resulting in a sense fusion of the *SHP2* enhancer to the ‘long’ 1040 bp *FBP1* promoter fused to the *GUS* reporter gene (pGD393). For the other constructs the *Sal*I-*Sal*I fragment of the cloned PCR *SHP2* enhancer fragment was first subcloned in pBluescript SK^+ vector (Promega) in both orientations (pARC012 and pARC013). The ‘long’ *pFBP1::GUS* fragment was also subcloned as an *Eco*RI-*Eco*RI from pFBP12E into pBluescript SK^+ vector in both orientations (pARC014 and pARC015). A *Cla*I-*Xho*I fragment containing the sense or antisense *SHP2* enhancer was then, inserted adjacent to the *pFBP1::GUS* fragment in both vectors pARC014 and pARC015. This resulted in an antisense fusion of the *SHP2* enhancer upstream of the ‘long’ *pFBP1::GUS* fragment and in vectors where the *SHP2* enhancer was downstream of this fragment in either sense or antisense orientation. The total inserts of these vectors were subcloned in the binary vector pBINPLUS® by using *Xba*I and *Kpn*I. All vectors were checked in detail by restriction sites and fragment length analysis.

GUS activity

Histochemical localization of GUS activity was performed as described by Jefferson et al. (1987).

Results

Tissue specificity of the *SHP2* enhancer

It has been shown previously that the *SHP2* gene is expressed in the dehiscence zone of *Arabidopsis* siliques (Savidge et al., 1995; Liljegren et al., 1998; Liljegren et al., 2000). To test whether a putative *SHP2* enhancer fragment contains the *cis*-acting elements and all its tissue specific regulating sequences, a 1220 bp upstream sequence was tested for its promoter/enhancer activity. This 1220 bp fragment was chosen –1275 bp till –55 bp upstream of the transcription initiation site, which was also 8 bp upstream of a putative TATA box. This 1220 bp *SHP2* promoter fragment contains a CarG-box like sequence, which is the putative *AGAMOUS* binding site and which is very likely to be important for its regulation. This promoter fragment (from now on referred to as the *SHP2* enhancer) was fused to a minimal *-47-35S* promoter, which was fused to the *GUS* reporter gene

(*GUSXX-47*) (Pasquali et al., 1994). This construct will allow the analysis of its transcriptional enhancer activity and tissue specificity. This binary vector (Figure 1A) and a control construct, which carries only the empty vector (*GUSXX-47*), were introduced into *Arabidopsis* plants ecotype Col-0. In table 1, the GUS expression patterns of 24 T1 plants that were analyzed are summarized. No GUS expression was observed in plants containing only the empty vector. In most of the GUS positive plants, GUS staining was observed in the dehiscence zone (Figure 1B). This expression in the dehiscence zone was seen at earlier stages during flower development (Figure 1C). Besides GUS expression in the dehiscence zone, some plants also displayed GUS expression in other tissue like in the pollen grains (Figure 1D), ovules (Figure 1E), funiculus (Figure 1F), nectaries (Figure 1G) and vascular junction at the receptacle (Figure 1H). Similarly to the results obtained here, *SHP1* and *SHP2* genes were previously described to have expression in other tissues like septum, ovules and funiculus (Ma et al., 1991), in addition to the dehiscence zone (Savidge et al., 1995; Liljegren et al., 1998; Liljegren et al., 2000). In some selected plants, no GUS staining was observed. Although no molecular analyses were performed on them, it is very likely that these plants were not transgenic or were not expressing the *GUS* gene properly (table 1). Based on these results, it can be concluded that the 1220 bp *SHP2* enhancer still contains the dehiscence specific regulatory sequences of the *SHP2* promoter.

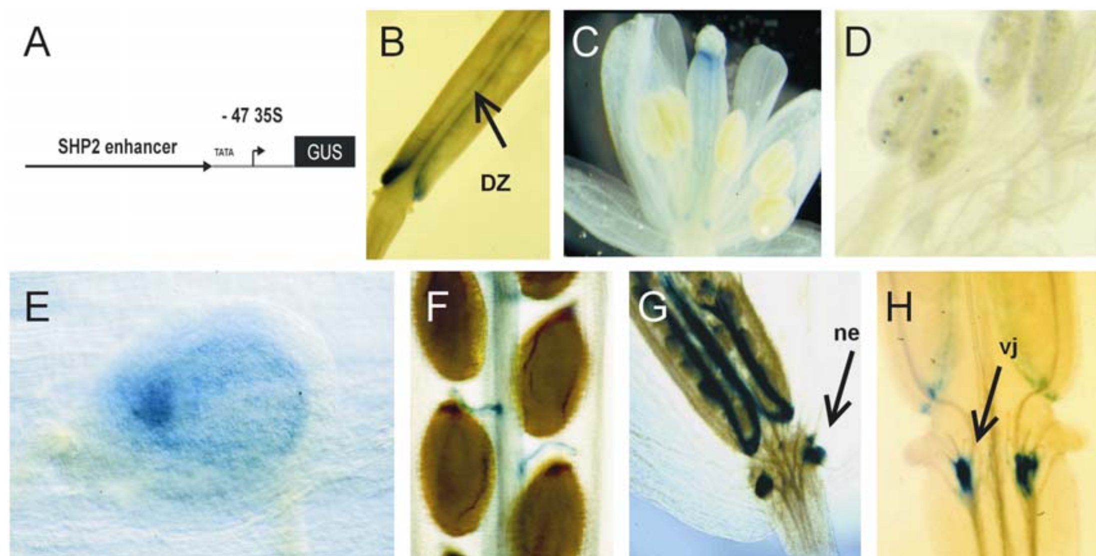

Figure 1. GUS expression pattern in *Arabidopsis* plants containing the construct *SHP2* fused to the minimal *35S* promoter (*-47-35S::GUS)*. **(A)** Schematic representation of the construct (which is not drawn on scale). **(B)** *Arabidopsis* silique showing GUS expression in the dehiscence zone (DZ, arrow). **(C)** A young flower with GUS expression in the DZ. GUS expression is also present in **(D)** polen grains, **(E)** ovules, **(F)** funiculus and **(G)** nectary (ne) and **(H)** vascular junction (vj) in the receptacle. ***Colour picture, see Appendix.***

Table 1. GUS expression of *SHP2* enhancer–*GUS* plants (pARC751).

Number of plants	Leaf	Sepals	Pollen	Ovules	Nectary	Funiculus	Carpels	DZ* young	DZ * mature
5 (21%)	-	-	-	-	-	-	-	-	-
10 (42%)	-	-	+	-	-	-	-	+	+
3 (12.5%)	-	-	+	+	-	-	-	+	+
3 (12.5%)	-	-	+	-	+	-	-	+	+
1 (4%)	-	-	+	+	+	-	-	+	+
1 (4%)	-	-	+	-	+	+	-	+	+
1 (4%)	-	+	+	+	-	-	+	-	-

/* DZ (dehiscence zone)

Activity of the *SHP2* enhancer

An enhancer is defined as *cis*-acting DNA sequences that can increase the transcription level of genes by binding to specific transcription factors. For full promoter activity also the DNA region directly upstream the transcription start site and containing the TATA box is required (Lewin, 2000). Enhancers usually can function in either orientation and separated from a minimal promoter domain. To obtain an enhancer for efficient tissue specific activation tagging, these features are essential. To test all these features of the *SHP2* enhancer, several constructs were generated (Figure 2).

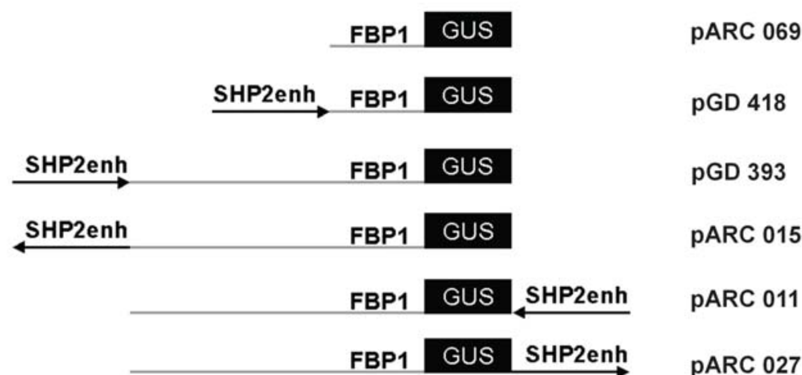

Figure 2. Schematic representation of the constructs in which the *SHP2* enhancer was fused to the *pFBP1::GUS* promoter-gene construct in different orientations. pARC069: the *pFBP1::GUS* construct used as control. pGD418: *SHP2* in sense orientation upstream of the short version of the *pFBP1* promoter. pGD393: the *SHP2* enhancer fragment is located upstream of the long *pFBP1::GUS version*. pARC015: the *SHP2* is also located upstream of the long *pFBP1::GUS* construct, however in antisense orientation. pARC011: the *SHP2* is placed downstream of the *GUS* gene in sense orientation and *SHP2* is placed in antisense orientation in the same long version of the *FBP1* promoter, in pARC027. This schematic representation is not drawn on scale.

Because enhancers are not able to act alone (Lewin, 2000), a minimal or natural promoter is necessary. Therefore, the *FLORAL BINDING PROTEIN1* (*FBP1*) promoter was chosen because this petunia promoter regulates expression in whorl 2 and 3 of the flower (petals and stamens respectively) (Angenent et al., 1993). In petunia two different lengths of the *FBP1* promoter fragment were analysed, a 220 bp 'short' promoter and a 1040 bp 'long' *FBP1* promoter. Both promoter fragments showed the same specificity and levels of expression in petunia (Angenent et al., 1993). As control, a binary vector harbouring the 'long' *FBP1* promoter sequence fused to *GUS* was introduced into *Arabidopsis* plants. Several transgenic plants were obtained with different levels of GUS expression in petals (often very weak) and filaments (stronger) (table 2 and figure 3A, B). This result indicates that this promoter acts as expected with the same specificity as in petunia (Angenent et al., 1993) and can be used to test the *SHP2* enhancer. Figure 2 summarises the vectors that were used to test the *SHP2* enhancer activity based on its ectopic expression in combination with the *FBP1* promoter. In the first two tester constructs, the *SHP2* enhancer was fused in sense orientation, with both the 'short' and the 'long' versions of the *FBP1* promoter resulting in pGD418 and pGD393 respectively (Figure 2). These constructs were used to test whether the *SHP2* enhancer fragment could ectopically activate GUS expression in combination with the *FBP1* promoter and moreover, whether the enhancer still keeps its tissue specific activity when combined with a natural promoter. By using the 'short and 'long' version of the *FBP1* promoter, the influence of the distance towards the promoter was analysed. The other three constructs should identify whether the *SHP2* enhancer acts independently of its orientation and position related to the coding sequence. The results of the GUS staining of the transgenic *Arabidopsis* plants expressing these constructs are summarised in table 2. In general, all constructs that contained the *SHP2* enhancer fused in either way to the *pFBP1::GUS*, showed besides the *FBP1* driven expression in worls 2 and 3 (Figure 3C), also ectopic GUS expression in the dehiscence zone (Figure 3D). In whorls 2 and 3 the GUS expression driven by the *FBP1* promoter was similar or in some lines slightly enhanced compared to the expression observed in plants that contained the control construct with only the *pFBP1::GUS* construct (pARC069). Although not quantified, we concluded from these visual observations that the *SHP2* enhancer did not substantially upregulate the *FBP1* promoter activity, but it had its action mainly in ectopic expression. This ectopic expression driven by the *SHP2* enhancer was mostly seen in the dehiscence zone tissue as expected. However in some plants, GUS staining in pollen grains was observed as well (data not shown).

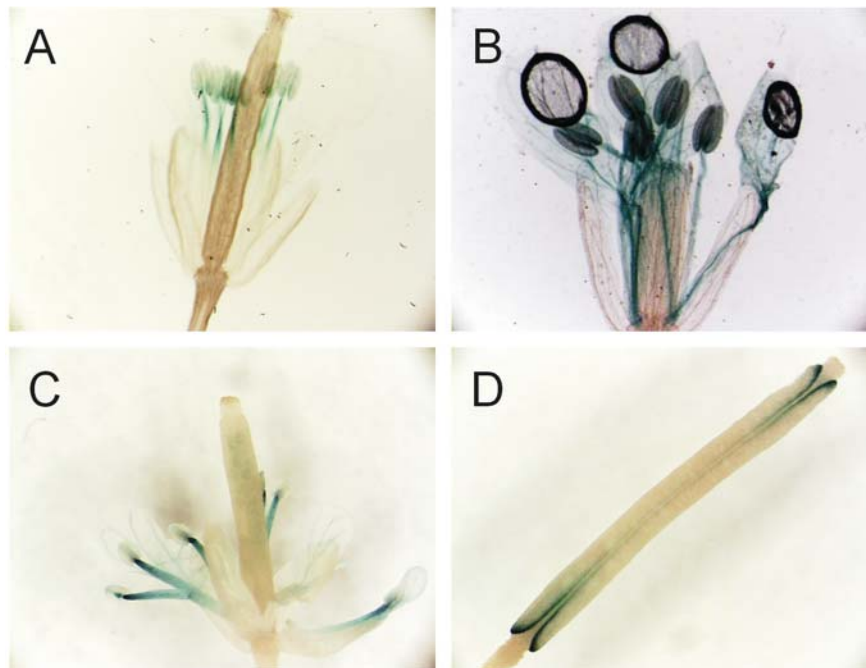

Figure 3. GUS expression pattern in *Arabidopsis* plants containing the *SHP2* enhancer inserted in the *pFBP1::GUS* construct (pGD418). **(A-B)** *pFBP1::GUS* (pARC069) flower, showing the *FPB1* driven expression in whorls 2 and 3 (petals and stamens, respectively). **(C)** Flower of a line containing pGD418 construct, showing GUS expression in petals and anther filaments and weak in the pistil **(D)** Silique of a pGD418 line. Besides the *FBP1* driven expression in petals and filaments, ectopic GUS expression is observed in the dehiscence zone. ***Colour picture, see Appendix.***

The distance of the *SHP2* enhancer relative to the promoter sequence did not seem to influence the frequency of plants showing ectopic expression in the dehiscence zone. When the *SHP2* enhancer is situated downstream of the *GUS* gene (pARC 011 and pARC 027) (Figure 2), the enhancer was still able to ectopically activate the expression with the same frequency as observed for the upstream sense position of the *SHP2* enhancer (Table 2).

The only construct that did not result in ectopic expression in dehiscence zone tissue was construct pARC015 (Table 2), which contains the *SHP2* enhancer positioned upstream, in an anti-sense orientation, linked to the "long" *pFBP1::GUS* fragment. Out of fourteen T1 plants analysed, 10 showed expression in whorls 2 and 3 only.

Table 2. GUS expression observed in tissues of transgenic *Arabidopsis* plants containing different constructs (see figure 2).

Name	Construct	Nº of plants	FPB1 expression			DZ*				
			P*	A*	F*	Young	Old	N*	O*	V*
pARC069	Long *FBP1::GUS*	7	+	+	+	-	-	-	-	-
		6	-	-	-	-	-	-	-	-
pGD418	*SHP2*enh sense Short *FBP1::GUS*	2	+	+	+	-	-	-	-	-
		2	+	+	+	+	+	-	-	-
		1	+	+	+	-	-	-	+	-
		1	+	+	+	+	+	+	+	-
		5	-	-	-	-	-	-	-	-
pGD393	*SHP2*enh sense Long *FBP1::GUS*	16	+	+	+	-	-	-	-	-
		13	+	+	+	+	+	-	-	-
		9	-	-	-	+	+	-	-	-
		5	-	-	-	-	-	-	-	-
pARC015	*SHP2*enh antisense Long *FBP1::GUS*	10	+	+	+	-	-	-	-	-
		4	-	-	-	-	-	-	-	-
pARC011	Long *FBP1::GUS* *SHP2*enh sense	2	+	+	+	-	-	-	-	-
		13	+	+	+	+	+	-	-	-
		4	+	+	+	+	+	-	-	+
		2	+	+	+	-	-	-	-	-
		7	-	-	-	-	-	-	-	-
pARC027	Long *FBP1::GUS* *SHP2*enh antisense	1	+	+	+	-	-	-	-	-
		3	+	+	+	+	+	-	-	-
		1	-	-	-	+	+	-	-	-
		1	-	-	-	-	-	-	-	-

/* DZ (dehiscence zone) P (petals); A (anthers); F (filaments); N (nectary); O (ovule); V (vascular bundle)

Discussion

Tissue specific activation tagging is a novel approach to generate mutants for a specific trait of interest. Requirements for such a strategy includes: i) tissue specificity of the used enhancer fragment, avoiding unwanted side effects in other tissues; ii) the activity of the enhancer should still be functional at least at several kbs from the natural promoter or gene increasing the efficiency that genes will be activated and iii) the insertion in the genome should be carried out by a system that minimizes the chances of complex insertion integrations (Nacry et al., 1998), which can cause silencing of the introduced enhancer (Chalfun-Junior et al., 2003).

Here we show that the 1220 bp *SHP2* promoter fragment taken from the upstream sequence of the *SHP2* gene contains most of the *cis*-regulatory sequences. It has been previously reported that the *SHP2* gene is expressed in the dehiscence zone of the *Arabidopsis* silique but also, in other tissues like septum, ovules and funiculus (Ma et al., 1991; Savidge et al., 1995; Liljegren et al., 1998; Liljegren et al., 2000). The *SHP2* enhancer sequence chosen in our study is mainly expressed in the dehiscence zone, although also GUS expression was also observed in ovules, funiculi and nectaries. The expression occasionally observed in a few pollen grains, nectaries and vascular junction at the receptacle were not described previously. We did not test GUS expression driven by the complete endogenous *SHP2* promoter in this same setup, which makes a comparison between published *in situ* hybridisation data and our data obtained with the enhancer studies more difficult. In conclusion, we demonstrated that it is possible to confer specific and ectopic expression of a particular gene by using an upstream enhancer sequence combined with minimal promoter elements.

The second prerequisite for an efficient activation tagging approach is that the enhancer activity should reach as far as possible from the minimal promoter elements (e.g. TATA box). Based on the results obtained with the constructs in which the *SHP2* enhancer was fused to the *FBP1* promoter (in combination with the *GUS* reporter gene), we detected in most of the combinations, ectopic expression by the *SHP2* enhancer. In some of the plants the GUS staining in the dehiscence zone was very strong and comparable with *–47-35S::GUS* transgenics. This was even the case when the enhancer was inserted downstream of the *GUS* gene, which resulted in comparable GUS expression levels as for the upstream sense positions.

It still remains unclear why construct pARC015 revealed no ectopic activation. This improper function may be specific for the *FBP1* promoter and therefore, it is not very likely that this is a general feature for an enhancer inserted in antisense orientation upstream a native promoter. Our results demonstrate that the enhancer activity acts over a distance of at least 2.0 kb, making it interesting to explore it in a tissue specific activation tagging strategy. When an even stronger activity would be required, it may be possible to create a tagging construct with a repeated *SHP2* enhancer like it has been performed for the *35S* enhancer (Weigel et al., 2000; Marsch-Martinez et al., 2002). However, this brings us to the third requirement for an efficient tagging strategy, i.e. avoidance of silencing, which could be induced by the insertion of repeated DNA sequences (Chalfun-Junior et al., 2003).

Based on the findings described by Marsch-Martinez et al. (2002) and our own observation on the methylation of T-DNA based activation tagging using the quadruple *35S* enhancer (Chalfun-Junior et al., 2003), we propose a transposon-based tagging strategy as being the most promising. Hopefully this strategy, in which the described *SHP2*

enhancer is used as tissue specific enhancer, will lead to a better understanding of pod dehiscence and to novel approaches to change the shattering behaviour of crops.

Acknowledgments

The authors thank John Franken for helping with sectioning and taking pictures, Stefan de Folter for helping at the beginning of the experiments. This work has been partially supported by CAPES-Brazil (BEX 1519/98-0).

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CHAPTER 4

Low frequency of T-DNA based activation tagging in *Arabidopsis* is correlated with methylation of *CaMV35S* enhancers

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Abstract

A powerful system to create gain-of-function mutants in plants is activation tagging using T-DNA based vehicles to introduce transcriptional enhancer sequences. Large *Arabidopsis* populations of individual plants carrying a quadruple cauliflower mosaic virus (*CaMV*) *35S* enhancer are frequently used for mutant screenings, however the frequency of morphological mutants remains very low. To clarify this low frequency we analyzed a subset of lines generated by this method. The correlation between the number of T-DNA insertion sites, the methylation status of the *35S* enhancer sequence and *35S* enhancer activity was determined. All plants containing more than a single T-DNA insertion showed methylation of the *35S* enhancer and revealed a dramatic decrease in *35S* enhancer activity. The results support the notion that in a large proportion of the T-DNA based activation tagged lines the *35S* transcriptional enhancer is silenced due to methylation, which is induced by multiple T-DNA integrations.

FEBS letters 555 (2003) 459-463

Introduction

At present many approaches are available to create genetic variants, like mutants, that may give clues about plant processes and their genetic control. Loss-of-function mutants are the most informative, because the mutant phenotype is directly associated with the biological function of the impaired gene. However, the use of loss-of-function mutants has its limitations, e.g. in the case of gene redundancy. This is a general phenomenon caused by genome duplications as is clearly present in *Arabidopsis* (*Arabidopsis* Genome Initiative, 2000), where the mutation of one gene copy often does not result in a detectable phenotype when other copies are still functional. An alternative approach for gene function analysis is gain-of-function mutagenesis, in which a gene is either ectopically or constitutively overexpressed compared to normal expression levels or patterns (Walden and Schell, 1994). Although in such a case a particular gene is not expressed in its normal biological context, it may give clues about how a gene affects certain biological processes. Ectopic expressions and overexpressions of individual genes have been performed for more than a decade and have provided valuable information about gene functions (Hayashi et al., 1992). An alternative to the gene-by-gene overexpression approach was developed by Walden et al. (1994), who designed a T-DNA based activation tagging approach to identify and isolate novel genes from tobacco. Only recently large-scale activation tagging gain-of-function mutagenesis was reported for *Arabidopsis*. Weigel et al. (2000) described the establishment of a large collection of *Arabidopsis* plants, transformed with a binary vector carrying a quadruple version of the cauliflower mosaic virus (*CaMV*) *35S* enhancer. This T-DNA activation tagging system has since then been used by many researchers and the obtained results illustrate the strength of the technology (Borevitz et al., 2000; Ito and Meyerowitz, 2000; Lee et al., 2000).

Although very successful, the frequency of dominant morphological mutants was surprisingly only around 1 in 1000 plants, which is much lower than can be expected considering the densely packed *Arabidopsis* genome and the estimated distance of up to 3 kb between an activated gene and the 4×*35S* enhancer (Weigel et al., 2000).

This relatively low frequency of T-DNA activation mutants is in contrast to recently published results obtained with a transposon based activation tagging system (Marsch-Martinez et al., 2002). The latter system, which generates single insertions of the activation tag, yields a frequency of about 1% morphological mutants, thus 10-fold higher than obtained with T-DNA based activation tagging (Marsch-Martinez et al., 2002).

Despite the low activation tagging frequency, the T-DNA based activation tagging approach is highly popular in *Arabidopsis* mutant screens (van der Graaff et al., 2000; Huang et al., 2001) with new populations being generated (Nakazawa et al., 2002). Nevertheless, with a low activation frequency, the method appears to be less attractive for a number of applications, for instance the use in species which cannot be easily

transformed in similarly large numbers as *Arabidopsis*. It will also be less suitable for more complicated or laborious *Arabidopsis* screens or for screens where only a few mutants are expected due to the specificity of the mutant class. We were therefore curious to discover the reason behind the low activation tagging frequency. The results described in this paper indicate that enhancer methylation and subsequent transcriptional silencing is a plausible explanation for the relatively low mutation frequency in T-DNA based activation tagging populations.

Materials and methods

Plant material

The four seed batches originated from set 2 of the `Weigel' collection, ecotype Columbia (Col-7) and were obtained from the Nottingham *Arabidopsis* Stock Centre, Nottingham, UK (http://nasc.nott.ac.uk/). Plants were grown in soil, under normal greenhouse conditions (22°C, 14/10 h light/dark). For selection on plates, seeds were surface sterilized by vapor phase seed sterilization (http://plantpath.wisc.edu/~afb/vapster.html) and selection with 10 mg l^{-1} hygromycin and 15 mg l^{-1} phosphinothricin-^{DL} (PTT) was applied on medium.

Southern blots

Genomic DNA was isolated from rosette leaves and approximately 300 ng of DNA was digested with restriction enzyme *Hin*dIII for the determination of number of T-DNA integrations. For methylation determination, genomic DNA was digested with the isoschizomers *Sau*3AI and *Mbo*I. DNA was electrophoresed in a 1.0% (w/v) agarose gel in 1×TBE (1.0 M Tris, 0.9 M boric acid, 0.01 M ethylenediamine tetraacetic acid (EDTA)) blotted onto Hybond N+ membrane (Amersham Pharmacia Biotech) following the normal instructions of the manufacturer. As probe, a fragment containing the *35S* enhancers was used and labeled by random oligonucleotide priming (Gibco BRL®).

Inverted repeat determination

Inverted repeats were determined by polymerase chain reaction (PCR), using a single primer designed 59 bp upstream of the 4x*35S* enhancers (PRO015), followed by a nested PCR with a primer overlapping the junction between the copies of the *35S* enhancer (PRO016). Visualization was performed by hybridization of the PCR product with a probe containing *35S* enhancer sequences. The following oligonucleotide sequences were used: PRO015: 5'-CGACTCACTATAGGGCGAATTGG-3' and PRO016: 5'-ATGTGATATCTAGATCCCCAACATGG-3'.

GUS activity determination

For GUS assay, protein extracts were prepared by grinding four just opened flowers, for each replication, in 50 µl extraction buffer (100 mM sodium phosphate pH 6.7, 10 mM EDTA, 0.1% Triton X-100, 0.1% sarcosyl, 10 mM dithiothreitol (DTT)), cleared by centrifugation for 5 min at 4°C, when MUG assay measurements were performed following previous description (Mlynárová et al., 1994).

Copy number level

The number of T-DNA integrations was determined by measuring the intensity of all hybridizing bands from a particular plant in the F1 Southern blot. These data were adjusted for unequal loading using the two internal standards, i.e. the bands representing the *35S::GUS* construct as a reference. The estimated number of T-DNA integrations were categorized into three groups of different copy number levels: from low, medium to high copy number levels. The F1 Southern blot was scanned with a Bio-Rad Molecular Imager® FX, and quantification analysis was done by Quantity One® software, version 4, Bio-Rad.

Results

Many T-DNA copies are present in activation tagging lines

We analyzed a small, randomly chosen, subset of plants from the T-DNA activation tagging population obtained from the *Arabidopsis* Stock Centre (Weigel et al., 2000). From four different seed batches (from pools of 96 plants), 12 T4 plants per seed batch were sown and labeled as follows: plants 1–12 from seed batch N23142, 13–24, from N23077, 25–36 from N23110 and 37–48 from N23190. A phenotypic analysis revealed one plant (#28) with an aberrant phenotype. This plant had a sterile, bushy and dwarfed phenotype and was unfortunately lost before enough tissue was collected for DNA isolation.

Southern blot analysis was performed for the remaining 47 independent T4 plants and a Columbia (Col-0) wild-type plant to get an idea about the number of T-DNA insertions, using *Hin*dIII as a restriction enzyme and *35S* fragment sequences as a probe. Among the 47 plants tested, 33 had a T-DNA integrated, with 26 apparently having multiple insertion sites and seven with single or low number of insertions (data not shown). From the 33 plants, four plants with many insertions (plants #10, 11, 20 and 38) and seven with low copy numbers (plants #2, 5, 15, 16, 21, 40 and 46) were chosen for further experiments.

Based on previous reports (Muskens et al., 2000; Matzke et al., 2001; Vaucheret and Fagard, 2001; Waterhouse et al., 2001) there may be a direct correlation between the number of T-DNA insertions and the methylation status of the T-DNA inserts, in particular when these are present as inverted repeats.

Other studies suggest that the complex T-DNA integration pattern often generated during plant transformations may trigger silencing mechanisms (Nacry et al., 1998; Tax and Vernon, 2001). In particular inverted repeats of T-DNA copies can induce either transcriptional gene silencing, through methylation of promoter sequences (Mette et al., 2000; Sijen et al., 2001), or posttranscriptional gene silencing causing a selective break-down of mRNA molecules (Kooter et al., 1999; Fagard and Vaucheret, 2000).

We hypothesized that methylation is the reason for the relatively low activation capacity of the *35S* enhancers used in the T-DNA activation tagged lines. To obtain evidence for this hypothesis the methylation status of the quadruple *35S* enhancer was determined. Southern blot analysis was performed with DNA digested with methylation sensitive and non-sensitive restriction enzymes *Sau*3AI and *Mbo*I, respectively, and with *35S* enhancer sequences as a probe. Except for plant #16, all plants showed methylation of the *35S* enhancer (data not shown).

A T5 offspring was raised from the 11 selected T4 plants for further analysis of the methylation and for the analysis of *CaMV35S* promoter activity. One T-DNA containing T5 plant derived from each T4 parent and a Col wild-type plant were crossed with a plant containing a single copy of the pCAMBIA1301 T-DNA carrying a *35S::GUS* construct (Roberts et al., 2003). F1 offspring plants were selected for the presence of both the activation tag T-DNA and the *35S::GUS* constructs (Figure 1A, B).

One F1 plant per cross was analyzed again by Southern blot to estimate the number of T-DNA insertions (Figure 2). At the same time, we determined the presence of inverted sequences of the T-DNA right border where the quadruple *35S* enhancer is located, in a PCR using one outward directed primer (Figure 3A). This analysis confirmed the previously estimated number of insertions except for plant #2, which now has a single integration locus of the T-DNA probably due to segregation of the T-DNA loci.

Methylation of the 4x*35S* enhancer silences a *35S* promoter in trans

Methylation of the quadruple *35S* enhancer was determined by Southern blot analysis of the selected F1 plants using methylation sensitive and non-sensitive restriction enzymes *Sau*3AI and *Mbo*I again and *35S* enhancer sequences as probe (Figure 3B). The fragments shared by all F1 plants, including the F1 with wild-type Columbia, represent the *35S* promoter present in the pCAMBIA1301 *35S::GUS* reporter construct. The *Sau*3AI/*Mbo*I sites from these fragments, which are used as internal controls, are located outside the *35S* promoter sequences and therefore are not likely to be susceptible to methylation (Figure 1B). The methylation analysis reveals that all plants except plant #16, which has a single `activation' T-DNA insertion, show detectable methylation of the *35S* enhancer, similar to previously determined for the T4 and T5 generations (data not shown).

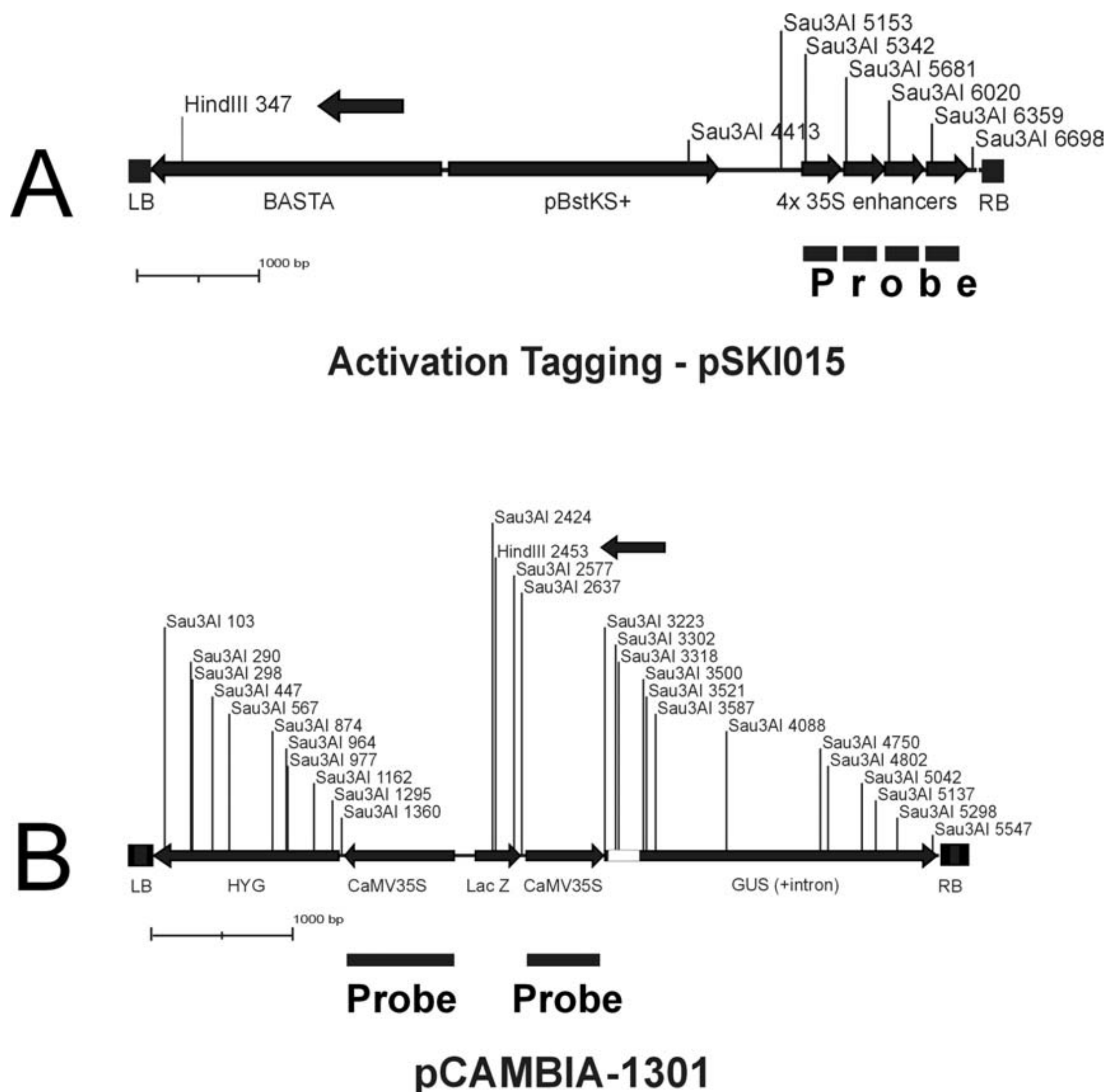


Figure 1. Schematic representation of the two T-DNA constructs used. Numbers shown with the restriction enzyme sites are the position where the respective enzyme cuts, counting from the left border (LB) towards right border (RB). **(A)** Activation tagging (pSKI015) T-DNA. To facilitate visualization, only the *Sau*3AI/*Mbo*I sites present in the *35S* enhancer region are depicted. The unique *Hin*dIII cleavage site is shown and highlighted by a black arrow. The four black square blocks are the regions used as probe, representing sequences of the *35S* enhancer. **(B)** pCAMBIA1301 T-DNA. All *Sau*3AI/*Mbo*I sites are shown. The unique *Hin*dIII cleavage site is shown and indicated by a black arrow. White box in the *GUS* gene represents the catalase intron. Two fat lines are the sequences used as probe (*35S* sequences).

This confirms our idea that the occurrence of multicopy T-DNA insertions frequently leads to methylation of the *35S* enhancer. Based on PCR amplification of the inserts (results not shown) we found that plants #11, 20, 38, and 40 contain an inverted repeat of the T-DNA. These plants show all high levels of methylation, suggesting a correlation between integration structure and methylation. In contrast however, a simple integration structure, e.g. in plant #2 does not always abolish methylation. It must be noted though that the F1 offspring of plant #2 originated from a T4 plant containing at least two T-DNA loci. An explanation therefore might be that the observed methylation is epigenetically inherited from the parental line (Mittelsten Scheid et al., 1998; Chareonpornwattana et al., 1999; Kakutani et al., 1999). Alternatively, it may be that occasionally a single integrated copy of the T-DNA is sufficient to trigger methylation of the enhancer, depending on the site of insertion (Montgomery and Fire, 1998; Stam et al., 1998; Stam et al., 2000).

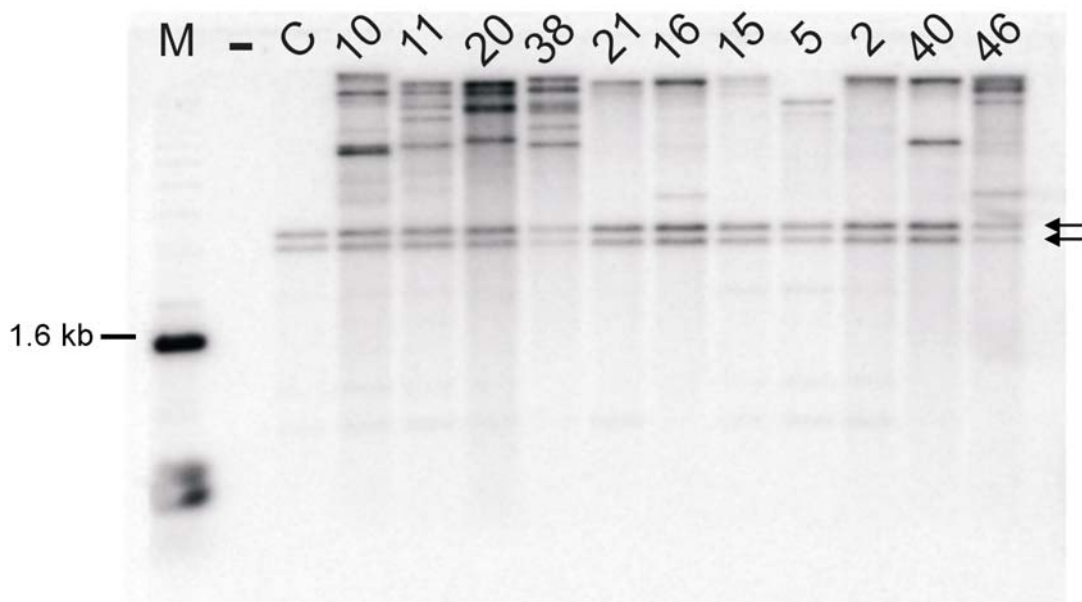
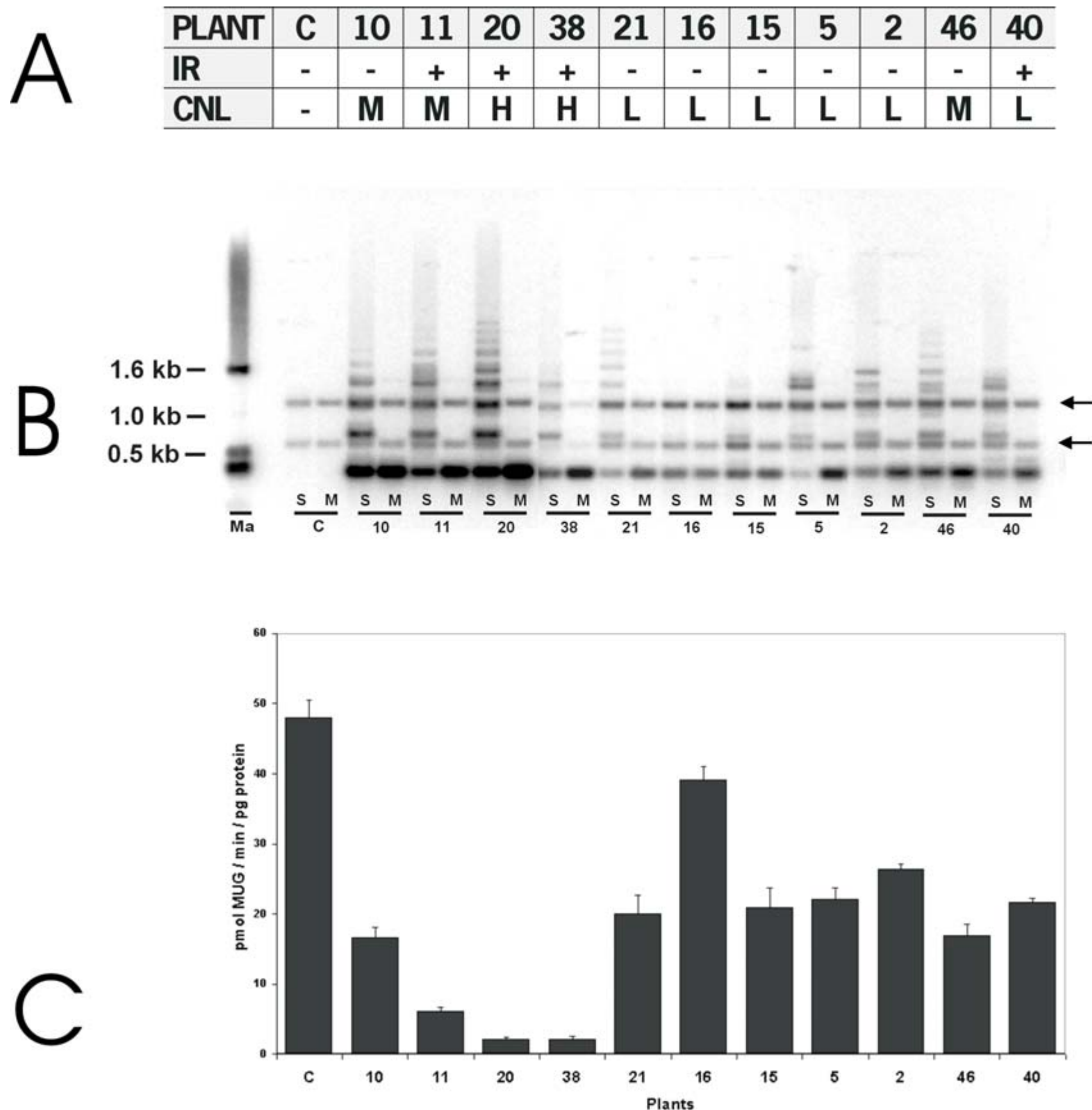


Figure 2. Southern blot analysis of the 12 F1 plants generated from the cross between 11 selected activation tagging lines (T5) and a plant homozygous for the *35S::GUS* construct. Genomic DNA was cut with *Hin*dIII and the blot was probed with *35S* sequences. The two fragments indicated with arrows are derived from the *35S::GUS* construct. C, control plant (F1 of Col-0×*35S::GUS*). M, marker lane.



PLANT	C	10	11	20	38	21	16	15	5	2	46	40
IR	-	-	+	+	+	-	-	-	-	-	-	+
CNL	-	M	M	H	H	L	L	L	L	L	M	L

Figure 3. Analyses of 12 F1 plants generated from the cross of 11 T5 activated tagged plants plus a wild-type plant (Col-0) with a homozygous plant containing a single *35S::GUS* insertion. F1 plants obtained from the cross between Col-0 and *35S::GUS* are used as control (C). T-DNA copy number levels, methylation status of the *35S* enhancer and *35S* promoter activity were determined. **(A)** The copy number level (CNL) of the T-DNA activation tagging vector as determined by Southern blot hybridization analyses. The number of T-DNA insertions was determined by measuring the intensity of the hybridizing bands derived from the activation tagging construct (see figure 2). The bands derived from the *35S::GUS* construct from the pCAMBIA1301 were used as internal control. The plants were categorized in three different `copy number levels'>five copies). Presence (+) or absence (-) of inverted repeats (IR) determined by PCR using a single primer designed 59

bp upstream of the 4×*35S* enhancers are indicated. **(B)** Methylation analysis of the F1 plants derived from a cross between activation tagged plants and a homozygous plant with a single copy T-DNA insert containing a *35S::GUS* construct (pCAMBIA1301). DNA was digested with *Sau*3AI (S) and *Mbo*I (M), a methylation sensitive and non-sensitive restriction enzyme, respectively. The two bands seen in control (C) represent the pCAMBIA1301 T-DNA insert, and can be used as a loading control. Ma, marker. **(C)** Expression of the GUS transgene in the F1 plants of crosses between 11 different activation tagging T5 plants and Col-0 with a *35S::GUS* plant. GUS expression was measured by a MUG assay (Mlynárová et al., 1994) on four just opened flowers. GUS activity levels were expressed as pmol of methyl-umbelliferone per min per µg of soluble protein (pmol MUG per min per pmol protein). Assays were repeated twice for each plant and the mean values are indicated as bars. The standard deviation of the mean is shown as thin line. C, control plant (F1 of Col-0×*35S::GUS*).

After that we showed frequent occurrence of methylation of the quadruple *35S* enhancer we were interested to see if this also had an effect on the activity of the enhancer, as was expected from transcriptional gene silencing experiments (Mette et al., 2000; Sijen et al., 2001). This was tested indirectly by measuring GUS activity in the same F1 plants used to estimate copy number level and methylation status (Figure 3C). All plants, except plant #16, show a significant reduction of GUS expression when compared to the control plant (C; Col×*35S::GUS*). There appears to be a negative correlation between methylation status and GUS activity. This result means that in the presence of methylated copies of the *35S* enhancer, a *35S* promoter (containing sequences identical to the methylated *35S* enhancer sequences) is silenced *in trans*. Probably the same transcriptional gene silencing mechanism, which caused methylation of the *35S* enhancer sequences, causes methylation of the *35S* promoter driving GUS expression (Mette et al., 1999). Although we did not directly measure silencing of the *35S* enhancer, we postulate that the observed methylation is sufficient to reduce the transcription enhancing potential of the quadruple *35S* enhancer.

Discussion

Silencing of the *35S* enhancer as a result of methylation is very likely the reason for the relatively low frequency of mutants found by T-DNA based activation tagging in the `Weigel' population (Weigel et al., 2000). We have shown in a representative selection of lines from this collection that the quadruple *35S* enhancer sequence is frequently methylated. The presence of the methylated *35S* enhancer sequences resulted in silencing of an in trans copy of a *35S* promoter, normally driving transcription of a *GUS* marker gene.

Several studies report that the presence of inverted repeats in complex chromosomal structures is the main reason for triggering methylation and subsequent silencing of the introduced sequences (Stam et al., 1998; Mette et al., 2000; Muskens et al., 2000; de Buck and Depicker, 2001). We observed relative high levels of methylation in plants with high numbers of T-DNA integrations especially when right border inverted repeats are present. Still, we did find plants in which no inverted repeat was detected, but in which the enhancer was methylated. For these plants other triggers of the methylation machinery could be active, such as the presence of direct repeats, DNA–DNA pairing of the enhancer region or the perception of repeated DNA as foreign (Matzke and Matzke, 1995).

Based on our observations, the T-DNA based activation tagging system might be improved by using a silencing impaired background. The methylation defective *ddm* mutants (Kakutani et al., 1999; Martienssen and Colot, 2001) are candidates for such an approach. Recently a number of other silencing defective mutants have been reported which could also be used (Mittelsten-Scheid and Paszkowski, 2000). As the regulation of transcriptional or posttranscriptional silencing is still not fully understood, probably a number of these mutants should be tried.

Another possibility is the use of single activation tag integrations. This strategy is followed in transposon based activation tagging (Marsch-Martinez et al., 2002), which has yielded plants with only a single copy of the activating construct. The relative high frequency of morphological mutants (approximately 1%) observed in the transposon based activation tagging population is in line with this single copy hypothesis.

A further improvement of the activation tagging system could be obtained by using an endogenous enhancer, which might be less susceptible to the silencing machinery than a foreign sequence such as the viral *35S* enhancer. This would also enable the use of tissue specific enhancers for the ectopic activation of gene expression in a tissue specific manner. The use of such enhancers has not been reported yet, but they can be attractive modifications to the T-DNA/*35S* enhancer based activation tagging as researchers are using it nowadays.

Acknowledgements

We thank Jan Peter Nap for his help with the MUG assay and useful suggestions and discussions. We also thank Maarten Koornneef for carefully reading the manuscript and stimulating discussions. This work has been financially supported by CAPES-Brazil (BEX 1519/98-0).

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CHAPTER 5

DOWNWARDS SILIQUES1, a member of the LATERAL ORGAN BOUNDARY family, controls petiole-blade boundary in *Arabidopsis* petals

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Abstract

The formation and the development of the floral organs require an intercalate expression of organ-specific genes. At the same time, meristem-specific genes are repressed to complete the differentiation of the organs in the floral whorls. In an *Arabidopsis* activation tagging population, a mutant affected in inflorescence architecture was identified. This gain-of-function mutant, designated *downwards siliques1* (*ds1-D*), has shorter internodes and the lateral organs such as flowers are bending downwards, similar to the loss-of-function *brevipedicellus* (*bp*) mutant. Cloning the affected gene in *ds1*-D revealed that *DS1* is a member of the LATERAL ORGAN BOUNDARY (LOB) family. Analysis of the loss-of-function mutant *ds1* did not show morphological aberration. Double mutant analysis of *ds1* together with *as2*, the *DS1* closest homolog, demonstrates that these two members of the LOB family act partially redundant to control cell fate determination in *Arabidopsis* petals. Moreover, molecular analysis revealed that overexpression of *DS1* leads to repression of the homeobox gene *BP,* which supports the model that an antagonistic relationship between LOB and homeobox members is required for the differentiation of lateral organs.

Introduction

During the past two decades, studies on the molecular control of flower development were very popular and successful. Enormous progress has been made in the understanding of reproductive organ development and the homeotic genes specifying organ identity. Nevertheless, due to its complex nature our knowledge is fragmentary and virtually absent when it concerns the differentiation of the floral organs.

An *Arabidopsis* flower is composed of four delineated organ types organised in symmetric whorls, which contain from the outermost to the innermost whorl four sepals, four petals, six stamens and two fused carpels. Flower formation is initiated in the floral meristem, where cells are initially undifferentiated (Weigel and Jürgens, 2002) and subsequently, their fate is determined by the action of homeotic genes belonging to the well-known ABC classes (Coen and Meyerowitz, 1991; Weigel and Meyerowitz, 1994). Floral organs, like leaves, are considered lateral organs, because they arise as differentiated structures from the flanks of an apical meristem. Lateral organs display polarity, which is determined by its initial relationship to the meristem, the apical or floral meristem. This polarity can be defined as adaxial-abaxial and proximal-distal. The adaxial side of the lateral organ anlagen is closest to the meristem, whereas the abaxial surface is more located to the periphery (Eshed et al., 2001; Tasaka, 2001; Bowman et al., 2002). Several genes are involved in the establishment of polarity either in leaves or in floral organs and they have been used to study cell polarity in *Arabidopsis* and other plant species. In *Arabidopsis*, *PHABULOSA* (*PHB*) and *PHAVOLUTA* (*PHV*) (McConnell et al., 2001) are key regulators of this process. Mutations in these genes alter cells of the abaxial surface into an adaxial identity. Members of the *KANADI* gene family are also important factors in abaxial cell fate, because both loss and gain-of-function mutants show strong aberrant phenotypes in abaxial cell identity (Eshed et al., 2001; Kerstetter et al., 2001). A double mutation in *FIL* (*FILAMENTOUS FLOWER*) and *YAB3* (*YABBY3*), both members of the YABBY gene family, leads to a more pronounced loss of polar differentiation of tissues in lateral organs, and the formation of abnormal meristems, which is attributed to the derepression of KNOX genes (Siegfried et al., 1999).

Genes of the *KNOTTED1* homeodomain, or KNOX family, such as *SHOOTMERISTEMLESS* (*STM*) and *BREVIPEDICELLUS* (*BP*; also known as *KNAT1*) are required for the maintenance and growth of the shoot apical meristem (SAM) (Bowman and Eshed, 2000) and they are downregulated when organ primordia develop (Semiarti et al., 2001; Byrne et al., 2002).

It has been reported that *ASYMMETRIC LEAVES 2* (*AS2*), a member of the recently described LATERAL ORGAN BOUNDARY (LOB) domain-gene family (Shuai et al., 2002), is important to repress the *KNOX* genes *BP* (*KNAT1*), *KNAT2* and *KNAT6* in leaves, by acting alone or in combination with *ASYMMETRIC LEAVES1* (*AS1*) (Semiarti et al.,

2001). *AS1* is a member of a small MYB-related gene family, which also contains the maize *ROUGH SHEAT2* (*RS2*) and the *Antirrhinum PHANTASTICA* (*PHAN*) gene. Expression studies revealed that these *AS1*-like genes are able to repress *KNOX* genes in leaves and by that they retain the differentiated state in the lateral organs (Schneeberger et al., 1998; Timmermans et al., 1999; Tsiantis et al., 1999; Byrne et al., 2000). The maize gene *RS2*, which is the putative orthologue of the *Arabidopsis AS1* gene, acts in the establishment of a blade-sheath boundary in maize leaves (Schneeberger et al., 1998). The *rs2* mutant displays disorganised differentiation of the blade-sheath boundary and furthermore, aberrant vascular patterning and the generation of semi-bladeless leaves (Schneeberger et al., 1998). In the *as1* mutant there are multiple bundles of elongated cells extending from the petiole into the leaf blade (Byrne et al., 2000).

Recent experiments indicated that *AS2* is involved in lateral organ polarity. Ectopic expression of *AS2* under the control of the *CaMV35S* promoter, resulted in adaxial-abaxial abnormalities in leaves (Lin et al., 2003), although in the *as2* loss-of-function mutant, polarity is not affected (Semiarti et al., 2001; Iwakawa et al., 2002; Xu et al., 2003). Changes comprise leaf lobbing and leaflet-like structures appearing on the leaves (Semiarti et al., 2001; Iwakawa et al., 2002; Xu et al., 2003). AS1 and AS2 are positive regulators of the founding member of the LOB family: the *LOB* gene (Byrne et al., 2002; Shuai et al., 2002). Based on its expression pattern, it has been postulated that *LOB* plays a role in the establishment of boundaries between the meristem and the differentiated lateral organs (Shuai et al., 2002). Other members of this LOB family have only been characterised by gain-of-function mutation (Nakazawa et al., 2003), which revealed remarkably similar phenotypes.

Here we describe the analysis of a gain-of-function mutant *downwards siliques1* (*ds1-D*) that was found in an activation tagging population. The phenotype of *ds1-D* is reminiscent with the phenotype of the loss-of-function *bp* mutant (Douglas et al., 2002; Venglat et al., 2002) and gain-of-function of a few members of the LOB family. Further analysis showed that this *bp*-like phenotype is due to the negative regulation of *BP* by the overexpression of *DS1*. In addition, mutant analysis of a knockout mutant of *DS1* revealed that this *LOB*-like gene acts redundantly with *AS2* (*LBD6*) in establishing boundaries in the *Arabidopsis* floral organs. A model describing the relationship between the *LOB* family members and the *KNAT1* gene will be discussed.

Material and methods

Screening activation tagging population

The stable *En-I* (*Spm*) transposon population (ecotype Wassilewskija (WS-3)) (Marsch-Martinez et al., 2002) was screened visually in the greenhouse for flower and silique aberrations. The selected mutant was selfed and a F1 segregating population was raised to check the inheritance.

Southern blot analysis

Genomic DNA from 24 F1 segregating plants and from the parental mutant was isolated (Pereira and Aarts, 1998) and approximately 300 ng of DNA was digested with restriction enzyme *EcoR*I. Equal loading of DNA was verified by ethidium bromide staining. DNA was eletrophoresed in a 1.0% (w/v) agarose gel in 1x TBE (1.0M Tris, 0.9M boric Acid, 0.01 M EDTA), blotted onto Hybond N+ membrane (Amersham Pharmacy Biotech) following the normal manufacturer's instructions. A 1.3 kb PCR fragment was used as probe, amplified from the 5' end of the *BAR* gene to the 3' end of the right transposon junction (Marsch-Martinez et al., 2002) and labelled by random oligonucleotide priming (Gibco BRL®).

Identification of the activated gene

To identify the putative activated gene, genomic DNA was used to isolate flanking DNA fragments of the *En-I* elements using a modified thermal asymmetric interlaced–PCR (TAIL-PCR) method, as previously described (Marsch-Martinez et al., 2002). Flanking DNA sequences were compared to the *Arabidopsis* database using BLASTN. The insertion was located between two genes, 2 kb downstream of gene At5g66860 and about 5 kb upstream of gene AT5g66870. The two predicted open reading frames were amplified using the following forward and reverse primers for At5g66860 and At5g66870, respectively: PRI839F: 5'-TGAGATGGCGAAATGGTGGC-3', PRI840R: 5'-AGACTTATTTATGCTTGAACAG-3', PRI841F:5'-AAATGGCGTCTTCAAGCTCTCCTTG-3', PRI842R: 5'-AGATTAACCAATGACATTCCTTCTACCC-3'. Expression was analysed by Northern blot hybridisation with approximately 5 µg of total RNA, isolated from rosette leaves (RL), cauline leaves (CL), young flower buds (top of the inflorescence) (CF) and open mature flowers (OF), from *Arabidopsis thaliana* plants, ecotype WS-3. RNA was isolated using the Qiagen® RNA isolation mini kit, denatured with 1.5M glyoxal, and fractionated on 1.4% agarose gel (w/v). The two genes described above were use as probes for hybridisation. The probes were labelled by random oligonucleotide priming (Gibco BRL®) and blots were hybridised and washed as described previously (Angenent et al., 1992). To test for equal loading, RNA quantities were checked by ethidium bromide staining of the gel.

Constructs

For the confirmation of the overexpressed gene, full-length cDNA of the predicted *DS1* gene (At5g66870) was cloned, using the GATEWAY™ cloning technology (Gibco BRL®). Approximately 0.2 µg of total RNA from closed flowers was used as template for the first RT-PCR reaction, following the supplier's instructions. The predicted *DS1* cDNA fragment was obtained by PFU proofreading polymerase (Stratagene), using gene specific

primers, above described as At5g66870Forw and At5g66870Rev. Terminal "A" ends were made with Super Taq polymerase (Stratagene) with approximately 1.5 units for 30' at 72 º C to be subcloned in pGEMT-EASY® (Promega). After subcloning in pGEMT-EASY®, a *DS1* cDNA fragment with GATEWAY™ sites (underlined) was obtained using the following PCR-primers: PRO060: 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTATGGCGTCTTCAAGCTCTCCTTGCGCAGCT-3' and PRO061: 5'-GGGGACCACTTTGTACAAGAAAGCTGGGTAGATTAACCAATGCAATTCCTTCTACCC-3'. Subsequently, the fragment was cloned into pDONR207 vector (Gibco BRL®) and recombined to the binary vector pGD625, establishing pARC082. The vector pGD625 was generated by cloning the *CaMV35S* expression cassette from pGD120 (Immink et al., 2002), as an *Asc*I/*Pac*I fragment in the blunted *Xba*I site of pBINPLUS (van Engelen et al.).

For the *pDS1::GUS* construct, the putative 1.8 kb *DS1* promoter fragment was amplified from genomic DNA of ecotype Columbia. PCR was carried out with TAQ plus precision polymerase enzyme (Stratagene) using the following primers including GATEWAY™ sites (underlined): PRO095: 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTCGCGTGAACGTGTCCTTATCATATAAGCAACC-3' and PRO096: 5'-GGGGACCACTTTGTACAAGAAAGCTGGGTTTTTTTTGTCTCTTTTCAGACTTGAGAGCCT-3', located just upstream of the ATG start. The obtained fragment was subcloned in vector pDONR207 (Gibco BRL®) and recombined to the binary vector PBGWFS7, resulting in pARC 201.

The *KNAT1* (*BP*) fragment that was used as probe was amplified using the following primers: PRO025: 5'-ATGGAAGAATACCAGCATGACAACAGC-3' and PRO026: 5'-GGCAGTCCAAGTAAGCTTGTAGGAGGG-3'.

Plant transformations

All constructs used were transformed to *Agrobacterium tumefaciens* strain GV3101 and introduced into *Arabidopsis* plants ecotype Columbia (Col-0) by the floral dip method (Clough and Bent, 1998). Selection for transformants was done on MS medium containing Kanamycin at 50 mg.L^{-1}, with seeds being surface sterilized (http://plantpath.wisc.edu/~afb/vapster.html), and resistant plants were transferred to greenhouse. For selection for BASTA resistance, seeds were vapor surface-sterilized (http://plantpath.wisc.edu/~afb/vapster.html) and grown directly on soil in the greenhouse. Seven days after germination (DAG) resistant plants were selected by spraying twice with a solution of 1:1500 (v/v) Finale® (Gluphosinate ammonium, 150g.L^{-1}, Aventis), with an interval of 2 days between the sprayings.

Knockout insertion mutants

The *ds1* mutant (WS-3) was obtained via the Wisconsin Facility Service (Sussman et al., 2000). The putative knockout mutant was confirmed via segregation analysis and also by PCR using gene specific primer PRI841 and T-DNA vector left border primer JL-270 (5'- TTTCTCCATATTGACCATCATACTCATTG-3'. This primer combination was also used for homozygosity identification. The insertion position was confirmed by sequencing the left flanking sequence of the T-DNA insertion (BigDye^{TM} sequencing kit, Applied Biosystems) using the JL-270 primer. It revealed that the insertion was located downstream of the predicted LOB domain, at approximately one third of the open reading frame.

T2 seeds of the *as2-1* mutant were obtained from the GABI-Kat FST population (Li et al., 2003), line ID 044C07, ecotype Col-0. Plants were grown under normal greenhouse conditions. To confirm the insertion in the gene, the following primer combination was used: PRO139 5'- TATAGTTTCTCATCACCAAGCGA-3' (*AS2*-specific) and T-DNA left border primer PRO140 5'- CCCATTTGGACGTGAATGTAGACAC-3'. The insertion in the open reading frame is located at amino acid position 40 of the predicted protein product, at approximately one third of the LOB domain.

Microscopy and histology

Scanning Electron Microscopy (SEM) was performed as described previously by Kater et al. (2000).

Histology and light-microscopy were performed as described by Angenent et al. (1993). Tissue preparation and histochemical staining (with phloroglucinol-HCL (1% (wt/vol) phloroglucinol in 6N HCL) for lignin detection were performed as described by Mele et al. (2003). Petals were cleared in Hoyer's solution as described by Liu and Meinke (1998) and examined as described by Angenent et al. (1993).

Results

Isolation of *downwards siliques1* (*ds1-D*): a dominant, gain-of-function mutant

To obtain flower developmental mutants, a population containing the *En-I* maize transposon-based activation tagging system (Marsch-Martinez et al., 2002), was screened for morphological mutants. A mutant affected in inflorescence architecture was identified and designated *downwards siliques1* (*ds1-D*) (Figure 1). In the *ds1-D* mutant, flowers and siliques bend downwards and the pedicels are reduced in length (Figures 1C-D). The inflorescence of *ds1-D* is more compact than wild type inflorescences due to a reduction of the internode length (Figures 1B and D). As a consequence of the reduced internode length, the overall plant height is approximately half the height of a wild type WS-3 plant.

Histological analysis of stems of *ds1-D* and wild type plants revealed a change in lignin deposition in the mutant. An irregular pattern of lignin accumulation resulting in

gaps in the lignin ring is observed in the mutant (Figures 1E-G). The lignin depositionalso started much earlier in *ds1-D* plants (results not shown). This difference in lignification depended on the position of the stems that were used for analysis, because sections at the basis of *ds1-D* stems revealed similar lignin deposition as observed for wild type stems (results not shown). The lignin deposition in *ds1-D* was comparable with the pattern present in the loss-of-function mutant *brevipedicellus (bp)* (Figure 1G).

To analyse the inheritance of the *downwards silique* trait, the *ds1-D* mutant was selfed and the progeny was analysed, revealing a 3:1 ratio between mutant and wild type phenotype. This demonstrated the dominant nature of the *ds1-D* mutant caused by a single transposon insertion.

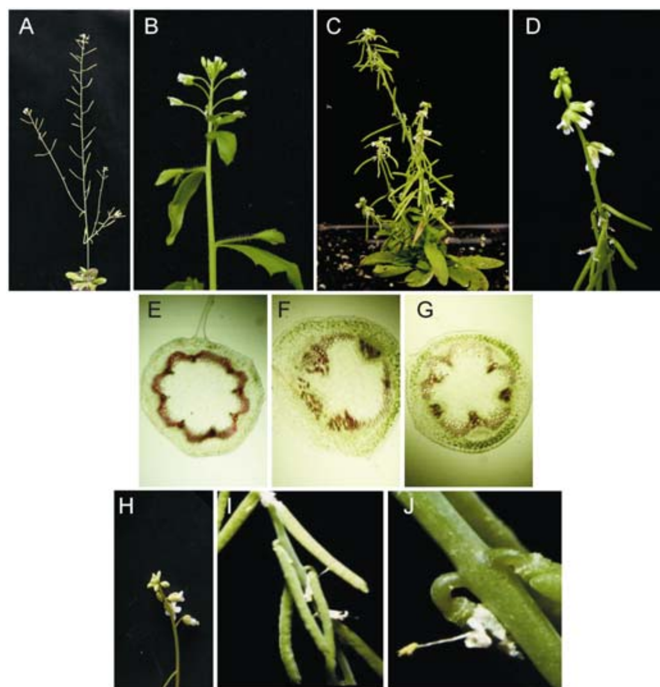

Figure 1. Comparison of phenotypes of the gain-of-function mutant *ds1-D* and wild type plants. **(A)** Adult wild type Col-O plant. **(B)** Inflorescence of a wild type plant. **(C)** *ds1-D* adult mutant plant **(D)** Inflorescence of the *ds1-D* mutant plant, with flowers and siliques bending downwards. **(E)-(G)** Histological analysis of fresh stem sections taken at the basis of the inflorescence at comparable developmental stages. The red/brown color represents lignin, which is deposited around the vascular bundles. **(E)** Wild type stem showing a thin continuous ring of lignin. **(F)** *ds1-D* mutant stem. Lignification in the *ds1-D* mutant is irregular and leaves gaps in the lignin ring. **(G)** *bp* mutant plant with a comparable lignification pattern as present in the *ds1-D* mutant. **(H)-(J)** Phenotypes of *35S::DS1* plants, which is similar to the *ds1-D* mutant phenotype. **(H)** Inflorescence of a *35S::DS1* line, showing flowers bending downwards. **(I)** Siliques of a *35S::DS1* plant. **(J)** Close-up of the internode region of a *35S::DS1* plant showing a short pedicel. ***Colour picture, see Appendix.***

Identification of the affected gene

The *ds1-D* mutant was obtained by transposon-based activation tagging using quadruple Cauliflower Mosaic Virus (*CaMV*) *35S* enhancers as activator (Marsch-Martinez et al., 2002).. First, Southern blot analysis was performed to determine whether single or multiple transposon insertions were present in the genome of the *ds1-D* plant. This segregation analysis confirmed that the mutation is caused by a single activation *I* element (AIE) (data not shown). Subsequently, TAIL-PCR was used to amplify the flanking DNA sequences of the insert (Liu et al., 1995; Liu and Whittier, 1995; Tsugeki et al., 1996). Sequence comparison of the obtained flanking regions with the *Arabidopsis* genome sequence, revealed that the 4x*35S* enhancers present in the AIE were positioned between two annotated genes, with a distance to the translation start sites of approximately 2 kb, for the upstream gene (At5g66860), and 5 kb for the downstream gene (At5g66870), respectively. A schematic presentation of the insertion in the genome is given in figure 2A. The 4x*35S* enhancers in the AIE are in the same direction as the predicted transcription of the upstream gene. Expression levels of the candidate genes were checked by RNA gel blot hybridisation, using tissues from rosette leaves (RL), stems (S), top of the inflorescence with young closed flowers (CL) and open mature flowers (OF) from mutant and wild type Col-0 and WS-3 plants (Figure 2B). Compared to the wild type samples, increased expression levels of the downstream gene (At5g66870) was observed in *ds1-D* mutant plants, suggesting that the overexpression of this gene in the mutant caused the *ds1-D* phenotype. Surprisingly, the expression of the upstream gene located approximately 2 kb from the *35S* enhancers appeared to be not affected in this mutant. This contrasts to previous reports where T-DNA activation tagging resulted in overexpression of the nearest gene to the 4x*35S* enhancers (Hayashi et al., 1992; Kardailsky et al., 1999; Weigel, 2000).

The *DS1* gene, which was overexpressed in the *ds1-D* mutant was previously annotated as the *LATERAL BOUNDARY DOMAIN36* (*LBD36*) / *ASYMMETRIC LIKE1* (*ASL1*) gene and encodes a novel cysteine rich protein with a so-called LOB domain (Iwakawa et al., 2002; Shuai et al., 2002). The LATERAL ORGAN BOUNDARY (LOB) domain is a conserved domain of 22 amino acids residues, which contains four conserved Cys residues ($CX_2CX_6CX_3C$) present in members of the *LOB* /*ASL* family. The *DS1* gene is very similar in amino acid sequence to *AS2* (known also as *LBD6*) (82% sequence similarity). A phylogenetic tree of the LOB family, including the close homologs *AS2* and *DS1* is depicted in figure 3. Using a T-DNA activation tagging approach, Nakazawa et al. (2003) described very recently a similar downwards siliques mutant phenotype obtained by overexpressing the same *DS1* (*LBD36*) gene.

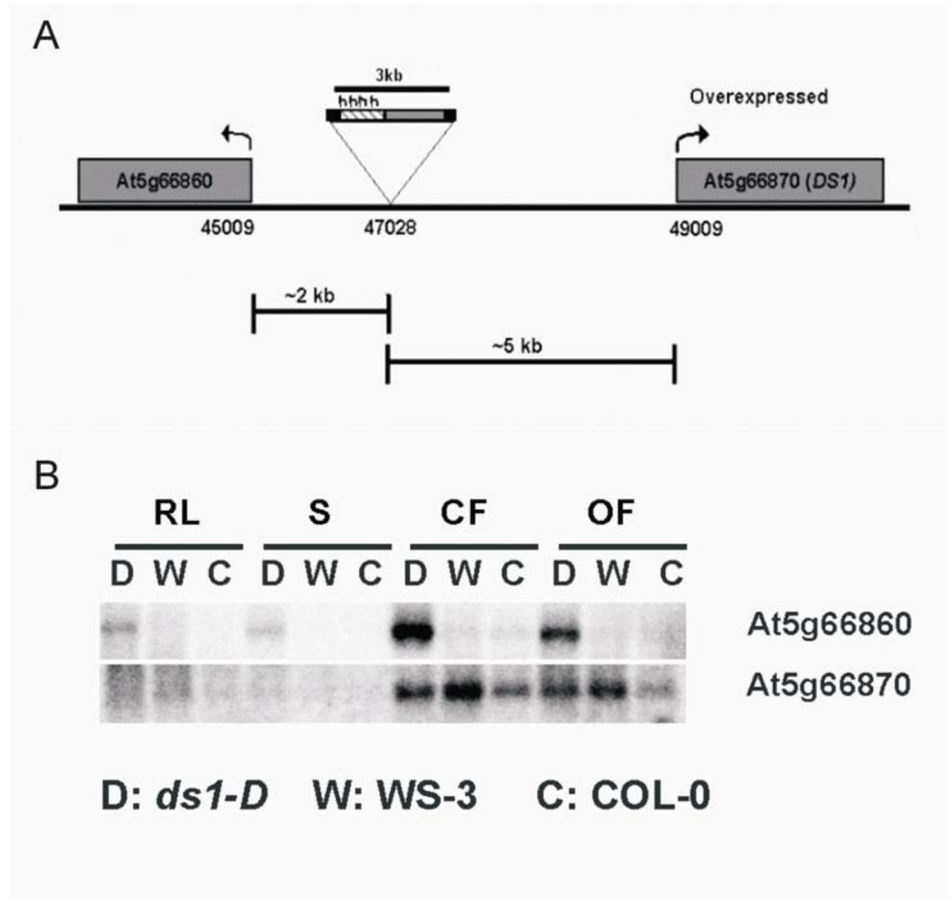

Figure 2. (A) Schematic representation of the insertion position of the activation I element (AIE) in the *ds1-D* genome. The insertion was located between two genes at chromosome 5. The distances from the transcription start sites of the genes to the 4x*35S* enhancer elements in the AIE are indicated in kilobasepairs (kb). Gray boxes depict the two genes and the arrows represent the direction of transcription. Numbers beneath the gray boxes show where the ATG start codon is positioned and the number beneath the AIE represent the insertion point in the genome. The AIE element is about 3.0 kb in length and is composed of the 4x*35S* enhancers (dashed box) and the *BAR* resistant gene (filled gray box). The black boxes are the left and the right junctions of the AIE element. **(B)** Northern blots showing the expression of the two genes that flank the AIE. The upper blot was hybridised with the upstream gene (At5g66860) and lower blot with the downstream gene (At5g66870). Samples were collected from rosette leaves (RL), stems (S), closed flower buds (top of inflorescence) (CF) and open flowers (OF) from *ds1-D* (D) plants and wild type WS-3 (W) and Col-0 (C) plants.

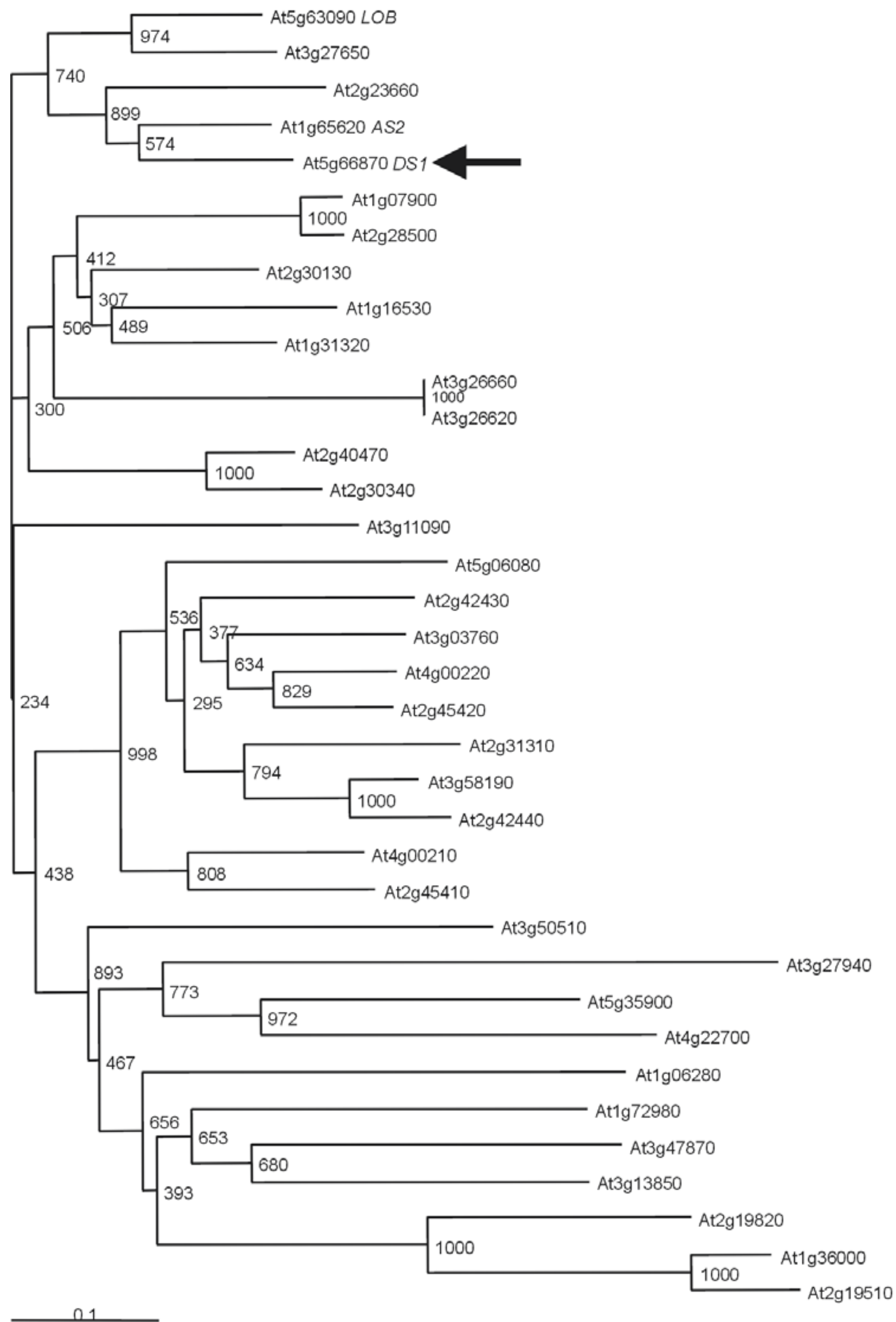

Figure 3. Phylogenetic tree of the LOB domain family in *Arabidopsis*. The comparison was done using the conserved LOB domain of all proteins as indicated by Lin et al. (2003). The tree was generated by the ClustalX program using the Phylip distance matrix with 1.000 bootstrap and the graphic representation was obtained by TreeView software, version 1.6.6

Confirmation of the phenotype

To confirm that the observed *downwards siliques1* phenotype was caused by the activation of the *DS1* gene, the predicted *DS1* cDNA was cloned behind the *CaMV35S* promoter. This *35S::DS1* construct was introduced in *Arabidopsis* wild type plants ecotype Col-0. From 10 Col-0 transformants, 9 exhibited the downward flower/silique phenotype, similar to *ds1-D* (Figures 1H-I). Among those 9 lines, one line showed increased severity in hyponastic rosette leaves compared to *ds1-D*. Two plants were smaller than the *ds1-D* mutant and another had a severe reduction in pedicel length (Figure 1J). In conclusion, the overall phenotype of the *35S::DS1* lines resembles the phenotype of the *ds1-D* mutant obtained by activation tagging, although some lines were more severe, most likely due to a higher *DS1* expression.

Expression analysis of *DS1*

To analyse *DS1* expression, a construct harbouring a 1.8 kb putative *DS1* promoter fragment fused to the β-glucuronidase (*GUS*) gene, *pDS1::GUS*, was introduced in *Arabidopsis* Col-0 plants. The GUS expression patterns observed were consistently in all GUS expressing lines (17 out of 22 lines) and are shown in figure 4. GUS expression was detected in many parts of the plant at various stages of development. Expression was observed in trichomes of mature rosette leaves and in cauline leaves. In young floral buds, the GUS activity was very strong in the distal part of the pistil at stages when style and stigma start to develop (Figures 4B, C). Most interesting, GUS activity was strongest at the base of many lateral organs, including branching points of the inflorescence and floral organs (Figures 4C, D and F). This expression pattern observed in many organ boundaries is similar to the result reported by Shuai et al. (Shuai et al., 2002) with an enhancer trap line showing expression of the *LOB* gene. GUS expression was also detected in pedicels, particularly those from older flowers and siliques, with a gradient of staining coming downwards from the abscission zone to the lateral axil (Figure 4E). In mature flowers, specific staining is visible at the base of petals and sepals, and progressively decreases towards the distal part of these floral organs (Figures 4F, G). Furthermore, GUS expression is detectable in ovules (Figure 4H), although this was only seen in high-expressing lines.

Knockout mutant of *DS1*

To get further insight into the function of the *DS1* gene, a loss-of-function mutant was identified by screening the Wisconsin population (Sussman et al., 2000). Homozygous mutant lines were generated and the insertion was confirmed by PCR analysis (data not shown). No visible mutant phenotype was observed in this *ds1* loss-of-function mutant, which suggests that *DS1* is redundant with other closely related genes from the LOB family. The most likely candidate for redundancy is *AS2* (Figure 3).

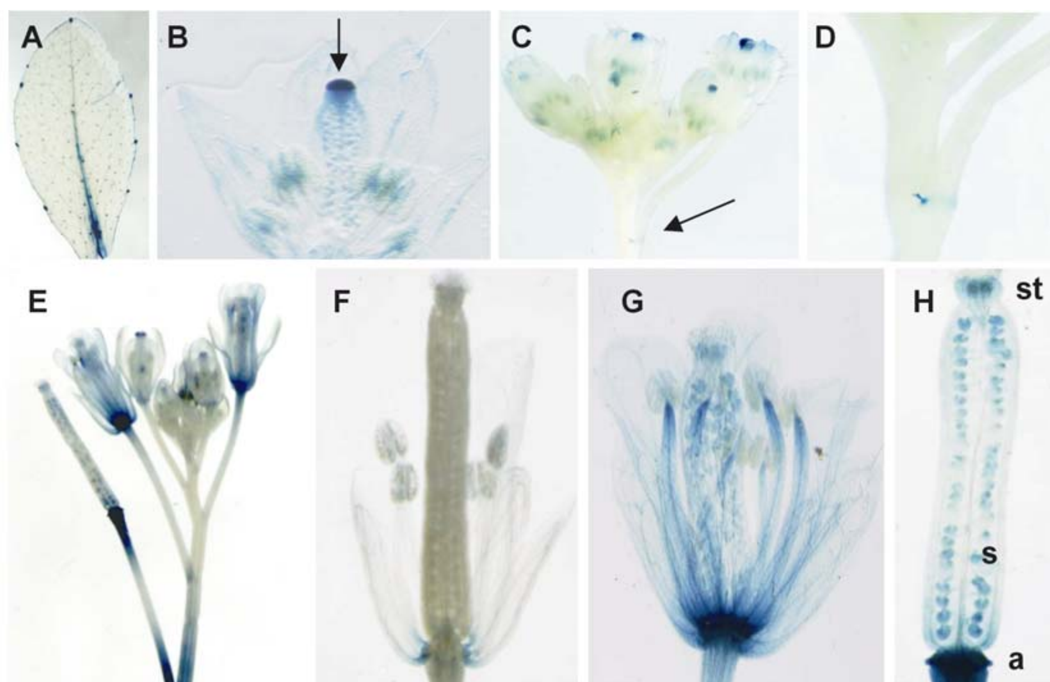

Figure 4. GUS expression analysis of *pDS1::GUS* plants. **(A)** Rosette leaf showing GUS expression in trichomes and the main vein. **(B)** Young floral bud with high GUS expression at the distal part of the pistil (arrow). **(C)** Inflorescence of a *pDS1::GUS* transgenic line with GUS staining in the style and at the base of lateral organs and branch points (arrow) **(D)** Close-up of (C) at the position of inflorescence branching. **(E)** Older inflorescence showing GUS expression in pedicels and floral organs. **(F)** Weak *pDS1::GUS* expressing line with expression at the basis of petals and sepals. **(G)** Mature flower of a strong *pDS1::GUS* expressing line. GUS activity is present at the basis of petals and sepals, in the anther filaments and ovules. **(H)** GUS expression in a young silique of the same line as shown in (G). st=stigma, s=developing seed, a=abscission zone. ***Colour picture, see Appendix.***

Therefore, double mutants with the *DS1's* closest homolog, the *AS2* gene, were generated. A T-DNA insertion line affecting the *AS2* gene was obtained from the GABI population (Li et al., 2003). Confirmation of the T-DNA insertion was done by sequencing the flanking DNA sequences. Plants homozygous for the T-DNA insertion in the *AS2* gene, as confirmed by PCR (results not shown), exhibit aberrations in rosette leaves, cauline leaves and sepals. The rosette leaves are lobed and curled downwards and have shorter petioles. Both the cauline leaves and sepals show serrations at the margins. These

phenotypic aberrations for the *as2* mutant have been described previously (Semiarti et al., 2001; Iwakawa et al., 2002). The *as2* mutant plants were used to generate a double mutant with *ds1*. Reciprocal crossings were made between *ds1* and *as2*. F2 segregating plants were analysed molecularly and phenotypically. No differences in progeny between the two populations generated by reciprocal crosses were observed, demonstrating that the phenotype is due to sporophytic trait. In the F2 population, three classes of phenotypes were segregating: plants with a wild type phenotype, mutants resembling the single *as2* mutant and lines with a stronger floral mutant phenotype. The genotypes of all plants were determined by PCR (results not shown) and revealed that the lines with the most dramatic phenotypic changes were all double mutants.

When comparing the *as2* single and *ds1 as2* double mutants, differences were observed in the floral buds (Figure 5). Wild type floral buds at around stage 6 are enclosed by sepals (Bowman, 1994). The buds remain closed until stage 13, prior to anthesis (Figures 5A, D). Petals become apparent just before that stage, when the sepals cannot cover the expanding petals completely (Figure 5J). At stage 6, when sepals should enclose the wild type buds completely, the *ds1 as2* double mutant plant, has narrower sepals resulting in exposed inner floral organs (Figures 5C, E, L). Another striking phenomenon, in addition to the exposed inner organs, is the outwards curling of the sepals and petals (Figures 5E, F, G, and H). In contrast, in wild type and single mutant flowers, the perianth organs are folded inwards and thereby, covering the reproductive organs perfectly (Figures 5B, G, H, I). The phenotype of premature flower bud opening has been described previously for *as2* (Ori et al., 2000; Byrne et al., 2002), although *as2* mutants grown under the conditions used in this experiment showed only mild defects in the flower (Figure 5B).

The wild type sepals overlap each other as can be seen in the scanning electron microscopic (SEM) illustrations (Figure 5I), while the overlap is absent in the double mutant flower buds from approximately stage 6 onwards (Figure 5K). The incomplete overlap of the sepals in the double mutant leaves a space between the sepals, allowing the inner organs to be exposed. The sepals and petals of the double mutants are slightly more elongated than in wild type and single mutant plants, which could also contribute to the phenomenon of early flower opening (Figures 5G, H). The SEM and macroscopic analyses also revealed a dramatic reduction in number of trichomes at the abaxial site of sepals of the double mutant (Figures 5I, K). Because the *as2* mutant is distinguishable from the double mutant, *ds1* does not contribute to the aberrations in the rosette and cauline leaves. The rosette leaves are lobed and they are also curled downwards. Cauline leaves are serrated and form lobes at the base (data not shown).

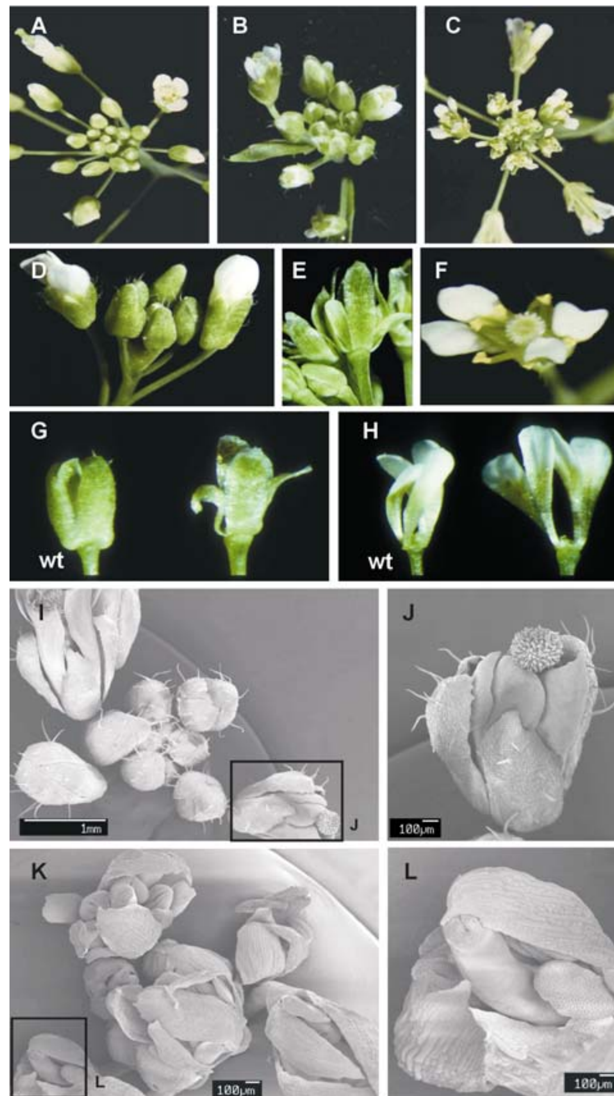

Figure 5. Phenotypes of wild type, *as2* mutant and *ds1 as2* double mutant plants**. (A)** Top view of a wild type inflorescence. **(B)** Top view of an *as2* inflorescence **(C)** Top view of a *ds1 as2* double mutant inflorescence **(D)** Side view of wild type inflorescence **(E)** Side view of the *ds1 as2* double mutant plant, showing that the flower buds are opened earlier. **(F)** A *ds1 as2* mutant flower depicting petals curling outwards and loss of symmetry. **(G)** Mature wild type (wt) and *ds1 as2* double mutant (right) sepals. The inner organs are removed. **(H)** Mature wild type (wt) and *ds1 as2* double mutant (right) petals. The other organs from the flower are removed. **(I)-(L)** SEM analysis. **(I)** Wild type inflorescence showing that flower buds are completely closed. **(J)** A wild type flower just before anthesis, when petals become apparent. The sepals are covered with trichomes **(K)** *ds1 as2* double mutant inflorescence showing that flower buds open earlier than in wild type **(L)** Close-up of a *ds1 as2* flower at floral stage 10, which is already opened and the small petals are exposed. ***Colour picture (A-H), see Appendix.***

To get more insight into the morphological abnormalities of the double mutant, histology of wild type and mutant flowers was examined (Figure 6). In a wild type flower at stage 10, the organs in each of the four floral whorls are symmetrically organised. The petals have the adaxial surface facing the inner organs, with a perfect concave shape (Figure 6A). In the *ds1 as2* double mutant, this concave adaxial petal shape is highly distorted: the form of the petal is irregular and spaces are present between the perianth organs (Figure 6B, C). It appears that the position of the four petals is not changed in the mutant. They are still alternating with the four sepals, thereby positioned in between the outer perianth organs. Occasionally, anther locule-like structures containing pollen are fused with a petal (Figures 6C, D).

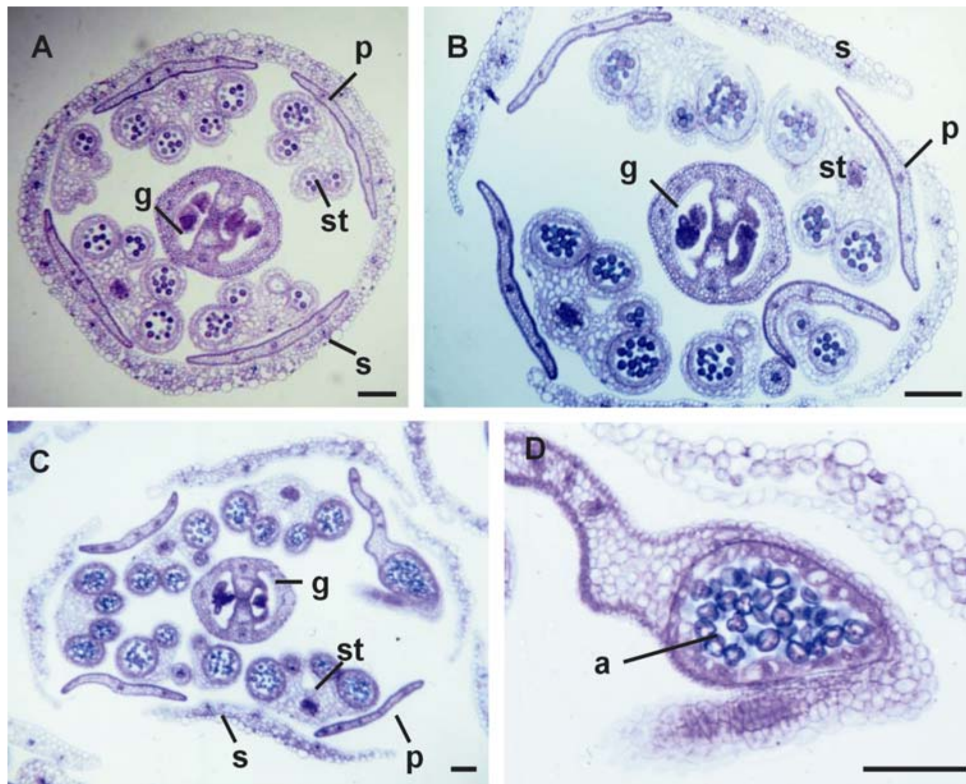

Figure 6. Light microscopic analysis of wild type and *ds1 as2* mutant flowers. The flower is composed of 4 sepals (s), 4 petals (p), 6 stamens (st) and a gynoecium (g). **(A)** Cross-section through a wild type flower. The flower bud is still fully enclosed by symmetrically organised sepals and petals. One stamen is missing in this section. **(B-C)** *ds1 as2* mutant flowers, showing that sepals are not enclosing the other inner organs. The petals are irregular in shape and position and are facing outwards. **(D)** Occasionally petals are fused to antheroid tissue containing pollen (a). Bars = 100 μm

Morphology of sepal and petal epidermal cells

Because changes in abaxial/adaxial symmetric has been reported for leaves of the *as2* mutant (Lin et al., 2003), the identities of the epidermal cells on both sites of the sepals and petals were monitored by SEM (Figure 7) and light microscopic (Figure 8) analyses. In the blade part of the wild type abaxial petal, epidermal cells are round shaped with irregular epicuticular ridges, whereas cells at the adaxial site are conical (Figure 7C, J; Figure 8C, K) (Bowman, 1994). The basal part of the wild type petal, which forms the petiole, has long elongated epidermal cells at both sides. The domain of these elongated cells spans about one third of the total size of a wild type petal. In the double mutant, conical cells are present at the adaxial site (Figure 7M) and irregular serrated cells cover the distal part of the petal at the abaxial side (Figure 7G), indicating that the abaxial/adaxial symmetry is not changed. However, in contrast, the proximal-distal organisation is affected. The elongated petiole-like cells are also present in the central part of the petal and even appear at the tip of the petal between normal round-shaped cells (Figures 7G, M; 8H, P). Due to this expansion of petiole-like cells into the distal portion of the petal, the boundary of the two cell types is shifted to approximately two-third of the petal size in the double mutant.

These changes in proximal-distal organisation may result in narrower and slightly longer petals as was observed for the *ds1 as2* mutant (Figure 5H). Furthermore, the outwards folding of the mutant petal could be explained by unequal expansion of the abaxial and adaxial sides.

Analysis of the epidermal cells of the sepals revealed less pronounced differences between wild type and double mutant (Figure 9). Obvious is the reduced number of trichomes at the abaxial side of mutant sepals (Figures 9 A, D). The epidermal cells are very similar at both sides of the sepal and a clear boundary between petiole and blade is missing. Nevertheless, ridges of elongated epidermal cells are present at the adaxial side of the mutant and are absent or shorter in wild type sepals (compare Figures 9C and 9G). These minor aberrations of sepal epidermal cells may reflect the difference in morphology of the mutant sepals when compared to wild type (e.g. see Figure 5G).

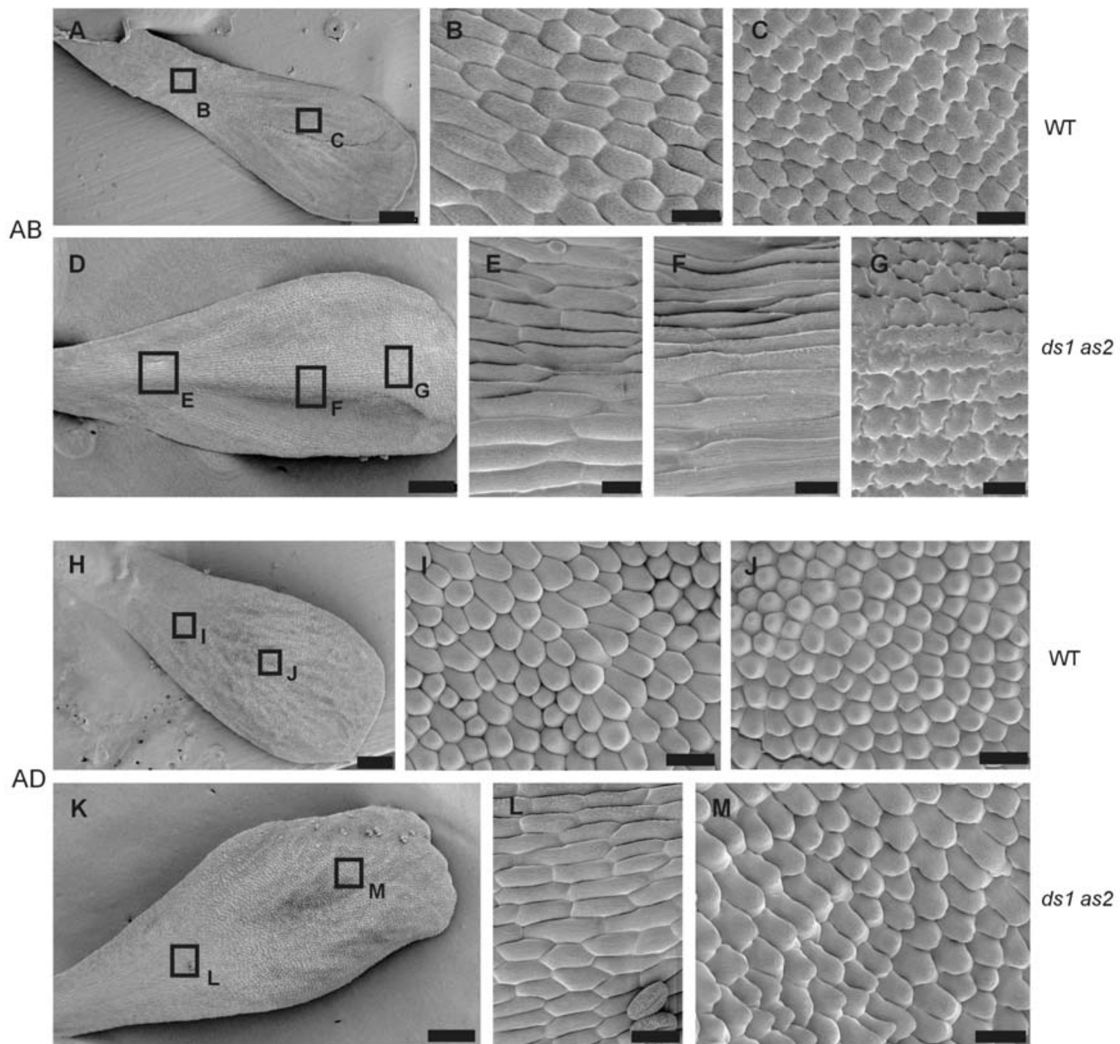

Figure 7. Scanning Electron Microscopy analysis of petal epidermal cells. The boxes in (A), (D), (H) and (K) indicate the position of the close-ups depicted in the other SEM figures. **(A-C)** Abaxial epidermal cells of a wild type petal. **(D-G)** Abaxial epidermal cells of a *ds1 as2* petal. **(H-J)** Adaxial epidermal cells of a wild type petal. **(K-M)** Adaxial epidermal cells of a *ds1 as2* petal. Bars in (A, D, H, K) = 200 µm; in (B-C, E-G, I-J, L-M) = 20 µm

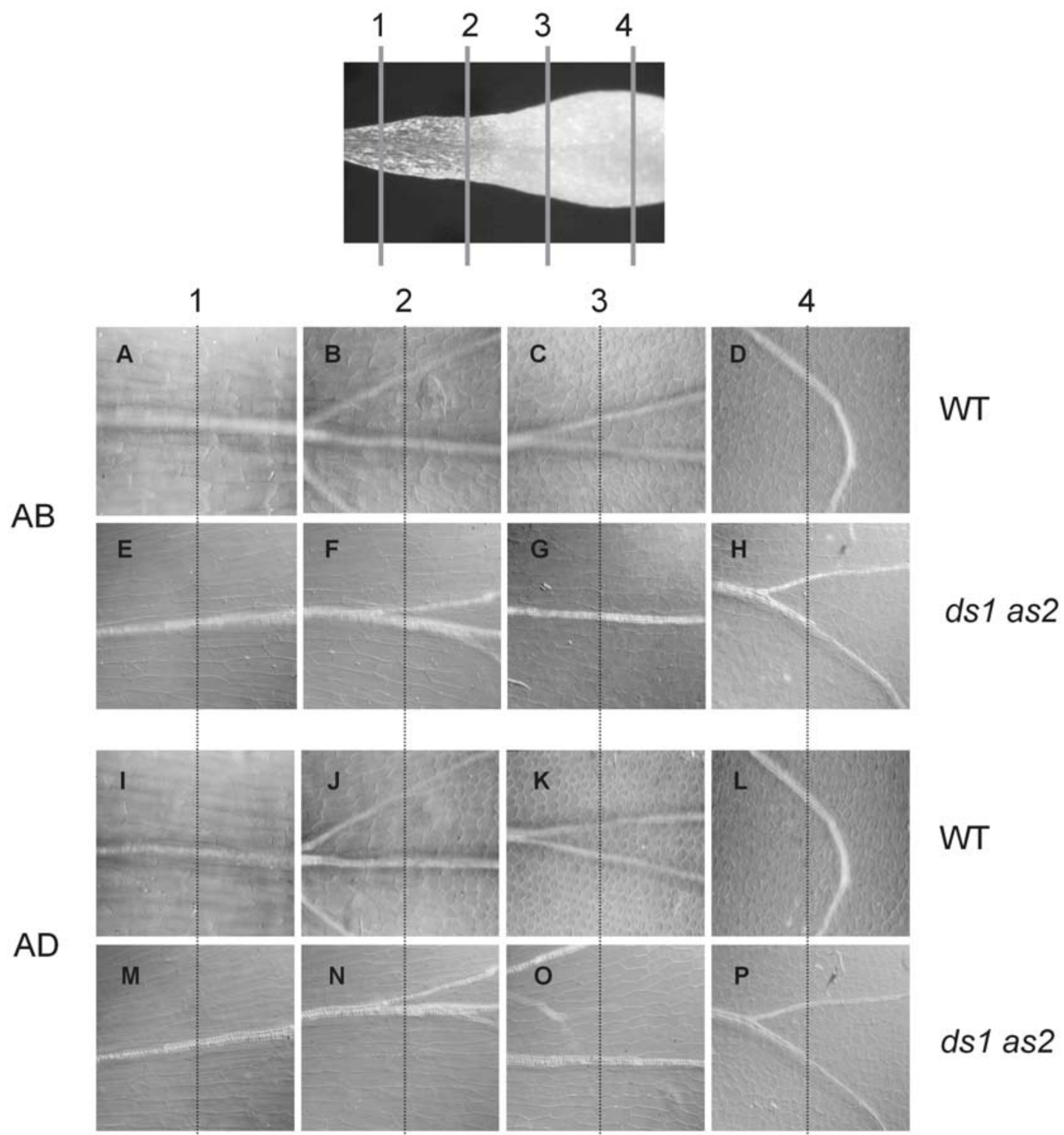

Figure 8. Light microscopic analysis of cleared wild type and *ds1 as2* petals. The petal is divided from basal to distal into 4 positions, 1-4 **(A-D)** Abaxial epidermal cells of a wild type petal. **(E-H)** Abaxial epidermal cells of a *ds1 as2* petal. **(I-L)** Adaxial epidermal cells a wild type petal. **(M-P)** Adaxial epidermal cells of a *ds1 as2* petal. Magnification is 400x in (A-P).

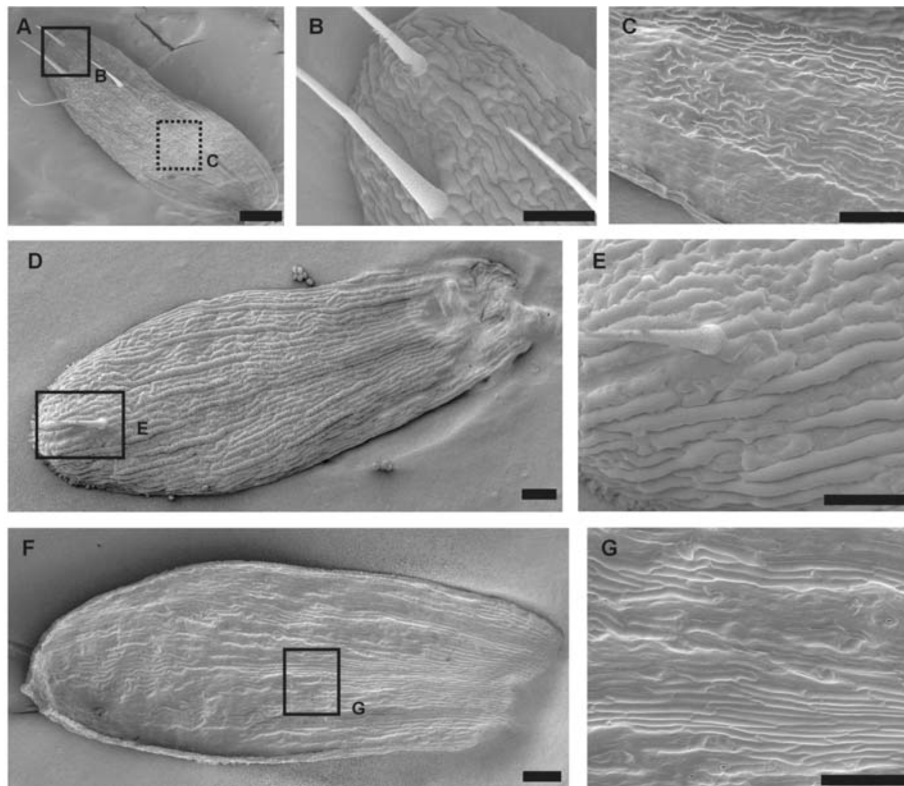

Figure 9. Scanning Electron Microscopy analysis of sepal epidermal cells. The boxes in (A), (D), and (F) indicate the position of the close-ups depicted in the other SEM figures. **(A-B)** Abaxial surface of a wild type sepal. **(C)** Adaxial surface of a wild type sepal at the position as indicated by a dotted box in (A). **(D-E)** Abaxial surface of a *ds1 as2* sepal. **(F-G)** Adaxial surface of a *ds1 as2* double mutant sepal. Bars in (A-G) = 100 µm.

Molecular interaction between *DS1* and *BREVIPEDICELLUS* (*BP)*

The *ds1-D* activation tagging mutant resembles the loss-of-function mutant *brevipedicellus* (*bp*), also know as *knat1* (Douglas et al., 2002; Venglat et al., 2002), suggesting that overexpression of the *DS1* gene suppresses the expression of the homebox gene *BP*. To provide evidence for this hypothesis, northern blot hybridisations were performed (Figure 10). Tissue samples from roots (R), rosette leaves (RL), cauline leaves (CL), young closed flower buds (CF), open flowers (OF), stems (ST) and siliques (S) were collected from *ds1-D* and *bp* mutants. The northern blots containing *ds1-D* and *bp* material were hybridised with *BP* and *DS1* specific probes, respectively. Based on the reciprocal expression patterns it can be concluded that there is an antagonistic relationship between these two genes. In the *ds1-D* mutant transcript levels of *BP* are dramatically decreased

compared to *BP* expression levels in wild type Col-0 tissues. In the young floral buds of *ds1-D* mutant, *BP* transcripts could not be detected. In the same blot low levels of *BP* expression were detected in mature open flowers and stems of wild type samples (Figure 10), which is in agreement with previously reported *BP* expression patterns (Lincoln et al., 1994). Furthermore, *DS1* expression is highly upregulated in the inflorescence of the loss-of-function *bp* mutant demonstrating the antagonistic relationship between the two genes.

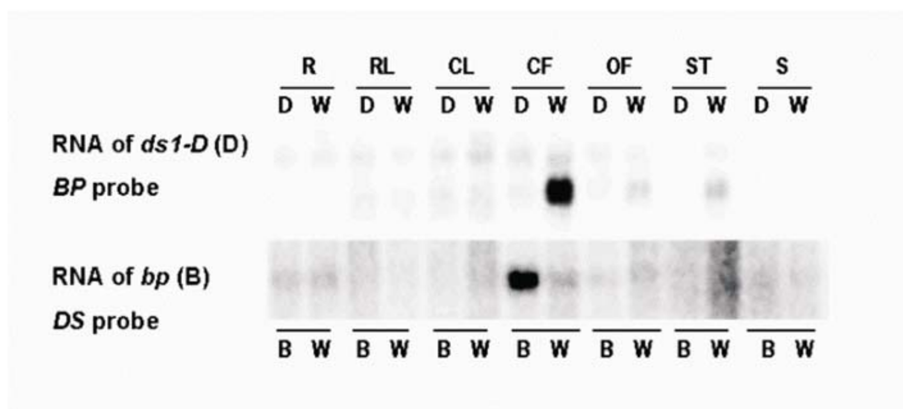

Figure 10. Expression analysis of *BP* and *DS*1 in wild type and mutant plants. Expression patterns of *DS1* and *BP* were examined in *bp* mutant and *ds1-D* tissues, respectively. Samples were collected from roots (R), rosette leaves (RL), closed flower buds (top of inflorescence) (CF), open flowers (OF), stems (ST) and siliques (S) from *ds1-D* (D), wild type WS-3 (W) and *bp* (B) plants.

Discussion

DS1 and the LBD gene family

DS1 is a member of the LATERAL ORGAN BOUNDARY domain (LBD) family, which was first described by Shuai et al. (2002) and consists of 42 members in the *Arabidopsis* genome. They all share the LBD domain with the founding member *LOB*. *LOB* is expressed at the base of the lateral organs in shoots and roots and is excluded from the floral organs (Shuai et al., 2002). In contrast, *DS1* appears to have a broader expression pattern, besides the expression at the axils, it exhibits expression in floral organs, the receptacle and style, suggesting that no redundancy exists between *LOB* and *DS1* in the flower. These two *LOB* genes overlap in expression mainly at the boundaries of the lateral organs, such as leaves and pedicels. *AS2*, the closest homolog of *DS1* and also known as *LBD6*, is expressed in almost all of the above ground parts of the plant except internodes

and pedicels (Iwakawa et al., 2002; Lin et al., 2003; Xu et al., 2003), demonstrating only a partial overlap in expression pattern between *DS1* and *AS2*. This is in line with what is thought to be a general mechanism how members of a large gene family may have evolved specific functions: duplication of the ancestral gene, followed by the modification of their expression patterns leads to diversification in gene functions (Ferrario et al., 2004).

The function of the conserved LBD domain is not known, although it has been suggested that this domain with a putative coiled-coil motif is involved in protein-protein interactions (Shuai et al., 2002). Recently, it has been reported that AS2 interacts with the MYB-domain containing protein AS1 in yeast two-hybrid experiments (Xu et al., 2003). This, however, does not exclude interactions between LBD family members, which is even very likely, because proteins with a similar amphipathic coiled-coil structure often interact with each other (Newman et al., 2000; Immink et al., 2002).

***DS1* suppresses *KNAT1* expression**

In the *ds1-D* mutant internodes are shorter and lateral organs such as flowers are pointing downwards. A very similar effect on inflorescence architecture was observed when the closely related gene *AS2* is overexpressed (Iwakawa et al., 2002; Lin et al., 2003; Nakazawa et al., 2003; Xu et al., 2003). This phenotype is reminiscent with knockout mutants of class 1 *KNOX* (*knotted-like* homeobox) genes, such as *KNAT1*, which is also known as *BREVIPEDICELLUS* (*BP*) (Ori et al., 2000). *bp* mutants are affected in internodes and pedicel development and show downwards pointing flowers and siliques (Douglas et al., 2002; Venglat et al., 2002). This is in line with its expression pattern being localised in the peripheral zone of the shoot apical meristem (Lincoln et al., 1994) and also in the cortical cell layers of the inflorescence stem and pedicel, but excluded from leaves and perianth organs (Douglas et al., 2002; Venglat et al., 2002). These phenotypes could be assigned to defects in differentiation of abaxial cells in the inflorescence axils, which demonstrates that *BP* is an important regulator of inflorescence architecture. We have shown that *DS1* down-regulates *BP* and, vice versa, *DS1* is upregulated in the *bp* mutant at the transcriptional level. Therefore, it is likely that the phenotype of the *DS1* gain-of-function mutant is due, at least partly, to the down-regulation of *BP*. A similar antagonistic relationship was also reported between *AS2* and several members of the *KNOX* family (Lin et al., 2003). This demonstrates that the closely related *LOB* genes, *LOB*, *AS2* and *DS1*, share the same role in down-regulating *KNOX* genes, although the differences in their spatial and temporal expression patterns determine where and when the *KNOX* genes are suppressed.

Ectopic expression of the *BP* gene give rises to lobed leaves and the formation of ectopic shoot meristems in the leaf blade, indicating that this *KNOX* gene mainly regulates adaxial/abaxial and proximal/distal polarity and differentiation zones (Ori et al., 2000). The

as2 loss-of-function mutant phenocopies the *BP* overexpression mutant, demonstrating again the antagonistic relationship between these genes (Chuck et al., 1996; Xu et al., 2003). In addition, overexpression of *AS2* by the *CaMV35S* promoter resulted in changes in abaxial and adaxial features of leaf epidermal cells, demonstrating that *AS2* is required for proper polarity determination.

In contrast, the *ds1* mutant does not show these changes in cell fate in the leaves, which is in line with its lack of expression in the leaves. In flower organs, where *DS1* is expressed, it may play a role in cell fate determination and suppression of *KNOX* genes. However, this role is not apparent in the *ds1* single mutant, because most likely it is masked by the action of a functionally redundant gene.

***DS1* and *AS2* acting together in proximal-distal symmetry determination.**

The *AS2* gene is the closest homologue of *DS1* and plays an important role in the establishment of normal leaf formation (Serrano-Cartagena et al., 1999; Semiarti et al., 2001; Iwakawa et al., 2002; Lin et al., 2003). *35S::AS2* plants display adaxial/abaxial changes in the leaves (Xu et al., 2003) and the formation of abaxial epidermal cells with a adaxial identity (Lin et al., 2003). In *as2* knockout mutants the polarity in petioles is affected, which is particularly obvious when the *as2* mutant is analysed in the L*er* genetic background (Xu et al., 2003). In flowers, the *AS2* mutation causes mild effects in the sepals: they are slightly curved outwards and are shorter with a partly serrated appearance (Ori et al., 2000). In addition, mutations in the *AS2* gene causes proximal-distal changes in leaves, which is also observed in mutants of the MYB-domain containing AS1 protein (Serrano-Cartagena et al., 1999; Sun et al., 2002). In particular in the *rough sheath2* (*rs2*) mutant, which is the maize equivalent of *as1*, the role of *RS2* in establishing proximal-distal polarity in leaves is apparent. In these mutants sheath cells expand in the distal region of the maize leaf blade without effects on dorso-ventral symmetry (Schneeberger et al., 1998; Tsiantis et al., 1999).

Whereas *ds1* loss-of-function mutants lack a visible phenotype, more pronounced aberrations were observed in the flower of the double *ds1 as2* mutants when compared to both single mutants. The flower buds opened precociously, which causes an exposure of the inner organs at early bud stages. These effects on the perianth organs could be attributed to an expansion of petiole cells towards the blade area, which causes a slight increase in length of the petals and outwards growth of sepals and petals. The phenotype observed in the *ds1 as2* double mutants indicates that there is partial redundancy between the two LOB members and that their roles in establishing proximal-distal polarity in the perianth overlap. The fact that the identity transformation of blade to petiole cells is subtle and not complete suggests that other members of the LOB family, e.g. *LBD10*, may play a role in this process as well.

LOB and *KNOX* genes in lateral organ development

Leaves of higher plants are produced through the differentiation of cells derived from the shoot apical meristem. Similarly, cells that form the floral organs undergo a comparable transition from floral meristem identity to differentiated state. Important factors in this transition are members of the *KNOTTED1-LIKE (KNOX)* homeobox family, such as *STM*, *KNAT1 (BP)*, *KNAT2*, and *KNAT6*, which are predominantly expressed in the meristematic cells and are suppressed upon differentiation in the lateral organs. Recent studies in *Arabidopsis* revealed a central role of a novel class of regulators, belonging to the LOB family, in the spatial control of *KNOX* gene expression. It has been shown that genes such as *LOB* and *AS2* are mainly active in leaves to control polarity, while in this report we have shown that *DS1* plays a role in differentiation of the perianth organs. Although it has been demonstrated here that *KNAT1(BP)* and *DS1* can act antagonistically, it does not mean that *DS1* is the one that excludes *KNAT1(BP)* expression from sepals and petals in wild type *Arabidopsis* flowers. However, Ori et al. (2000) showed that *KNAT1(BP)* is ectopically expressed in the floral organs in an *as2* mutant background. Based on this and the functional redundancy between *AS2* and *DS1* reported here, *DS1* might do the same.

The role of the MYB-gene *AS1* in this regulatory pathway has been postulated by Xu et al. (2003). The physical interaction between AS1 and AS2 proteins, forming a potential heterodimeric transcription factor may directly or indirectly control *KNOX* expression and promote cell differentiation in leaves. Also DS1 interacts with AS1 in yeast two-hybrid studies, which corroborates our finding that DS1 and AS2 are partly redundant (A. Chalfun-Junior; R.Immink; G.C.Angenent, unpublished data).

How adaxial-abaxial symmetry is promoted in sepals and petals remains to be elucidated, although it is very likely that LOB family members are essential in that process as well. Functional characterization of more *LOB* genes and defining their partial overlapping roles in lateral organ development will provide new insights into floral organ differentiation and underlying regulatory networks.

Acknowledgements

We are indebted to members of Gerco Angenent's lab for all contributions during the period of this work and Faye Rosin for comments on the manuscript. We would like to thank Rafaella Greco for help with the activation tagging population and Asaph Aharoni, Maarten Koornneef and Mark Aarts for helpful discussions. The authors also express gratitude to the Wisconsin *Arabidopsis* Facility for the *ds1* mutant. The *as2* T-DNA mutant was generated in the context of the GABI-Kat program and provided by Bernd Weisshaar (MPI for Plant Breeding Research; Cologne, Germany). We like to acknowledge Huub

Geurts for help with the SEM analysis. This work has been financially supported by CAPES-Brazil (BEX 1519/98-0).

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CHAPTER 6

NEEDLE1, a HELIX-LOOP-HELIX transcription factor involved in *Arabidopsis* carpel development

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Abstract

A phenotypic screening for pistil/silique mutants using an activation tagging population yielded the *needle1-D* mutant. Overexpression of *NEEDLE1* (*NDL1*) by the 4x*35S* enhancers present in the transposable element altered the normal formation of valve tissues in *Arabidopsis* siliques. The phenotype of the *ndl1-D* mutant resembles the phenotypes of mutants involved in the regulation of carpel development, such as *ettin*, *pinoid* and *pin-formed*. *NDL1* was cloned and encodes a member of the basic/Helix-Loop-Helix family of transcription factors. Expression analysis supports the notion that *NDL1* is involved in gynoecium development. Moreover, auxin levels in the *ndl1-D* are alterred suggesting that it plays a role in auxin transport during carpel development program in *Arabidopsis*.

Introduction

The female plant reproductive organ, the gynoecium, is a highly specialised plant organ that has long attracted the attention of scientists. In *Arabidopsis thaliana*, the gynoecium is composed of two fused carpels, separated by a septum (Ferrandiz et al., 1999). The septum is formed by the postgenital fusion of two outgrowths originating from the regions of carpel fusion (Bowman et al., 1999). At maturity, the gynoecium can be divided into the apical stigma, a short style, and a basal ovary containing the developing ovules (Bowman, 1994; Ferrandiz et al., 1999). After ovule fertilisation, the ovary develops seeds, then fruit elongates and differentiates into distinct cell types followed by maturation and seed dispersal.

The identity of carpels and the other floral organs is specified by three separate classes of homeotic genes, A, B and C, which act either alone or in combination (Coen and Meyerowitz, 1991; Weigel and Meyerowitz, 1994). More recently two additional classes of homeotic factors (D and E) were added to this model describing floral organ development (Theissen and Saedler, 2001). The class C gene *AGAMOUS* (*AG*) and E genes *SEPALLATA1/2/3* are involved in carpel identity determination. For the further development and differentiation of the pistil more genes are involved: among others, *SPATULA (SPT)* and *CRABS CLAW (CRC)* have been identified as important components of the carpel developmental program (Alvarez and Smyth, 1999). *SPT* is a member of the basic/Helix-Loop-Helix (bHLH) family of transcription factors (Heisler et al., 2001), while *CRC* encodes a YABBY-like transcription factor (Bowman and Smyth, 1999). *spt* mutants specifically disrupt carpel fusion and transmitting tract production (Heisler et al., 2001), whereas *crc* develops a wider and shorter gynoecium with the two carpels unfused at the apex (Alvarez and Smyth, 1999; Bowman and Smyth, 1999). These genes can act independently in gynoecium development; however, there is evidence for overlap in function (Alvarez and Smyth, 2002). In the double mutant *spt crc*, carpels are less fused and more separated, having almost no style or stigmatic tissues (Alvarez and Smyth, 2002). When those genes are combined with *ap2 ag*, a quadruple loss-of-function mutant, the remaining gynoecium structures are lost demonstrating that rather than being a direct downstream target of *AG*, *CRC* and *SPT* play a role in parallel with *AG* to specify the carpel developmental program in *Arabidopsis* (Alvarez and Smyth, 1999).

Recently it has also been shown that *AG* is redundant with the *SHATTERPROOF (SHP)* genes (*SHP1* and *SHP2*) in promoting carpel development. Moreover, a fourth gene, *SEEDSTICK (STK),* is involved and when the complete *AG* clade is nonfunctional, neither carpel nor ovules are formed (Pinyopich et al., 2003). This redundancy shows the complexity of factors involved in the gynoecium developmental process. Increasing the complexity, another important gene has been demonstrated to play a significant role in the gynoecium's destiny. *ETTIN (ETT*) encodes a transcription factor that shares a DNA-

binding domain with the auxin response factor (ARF) family of transcription regulators (Sessions and Zambryski, 1995; Sessions et al., 1997; Nemhauser et al., 2000). The founding member of this family, ARF1, was identified by its ability to bind to the auxin response element (AuxRE), which are found in the promoter region of auxin-responsive genes (Ulmasov et al., 1997; Ulmasov et al., 1999a). It has been shown that *ETT* plays a role in the patterning of the gynoecium primordia in a dose-dependent manner (Sessions et al., 1997). In the *ett* strong allele, there is just a thin stalk-like structure instead of the normal valve tissues of the pistil (Sessions and Zambryski, 1995). Sessions and coworkers (1997) proposed that the morphological defects found in *ett* mutants are caused by changes in regional boundaries positioned at the apical and basal limits of the valves, which results in an extensive loss of valve tissues and a gain of flanking tissues. In this case, there is a basalisation of the apical domain of the gynoecium (Sessions and Zambryski, 1995). ETT functions as a regulator of auxin responsive genes in gynoecium development (Nemhauser et al., 2000). Because of the presence of many auxin response elements in the *SPT* promoter region, it has been proposed that ETT may regulate *SPT* during gynoecium development by inhibiting *SPT* transcription (Heisler et al., 2001).

A similar stem-like gynoecium as formed in *ett* mutants is also seen in other mutants involved in auxin-regulated processes, such as the *pin-formed* (*pin*) and the *pinoid* (*pid*) mutants (Bennett et al., 1995; Christensen et al., 2000; Benjamins et al., 2001). In *pin* mutants, auxin flux is defective, which influences the determination of floral organs and causes a drastic loss of valve tissues in the gynoecium (Okada et al., 1991; Bennett et al., 1995). In strong *pin* mutants, flowers are completely missing and the inflorescences terminate in pin-like structures. *pid* mutant plants show a less severe phenotype, producing more flowers, but also in this mutant polar auxin transport is affected (Christensen et al., 2000; Benjamins et al., 2001). Exposure of wild type flowers to polar auxin transport inhibitors results in a gynoecium similar to that seen in *pin* and *pid* mutants, demonstrating the relationship between auxin transport and the *PID/PIN* genes (Bennett et al., 1995).

A novel mutant, designated *needle1-D* mutant (*ndl1-D*) was identified by screening an activation tagging population. The phenotype of *ndl1-D* mutants resembles mutants involved in the regulation of carpel development, such as *pin*, *pid*, and *ett*. To gain more insight in this complex and intriguing plant developmental program, the *NDL1* gene was cloned and belongs to the basic/Helix-Loop-Helix (bHLH) transcription factor family. Expression analysis suggests that *NDL1* may be involved in gynoecium development and its further relationship with known genes involved in the carpel developmental pathway may shed new light on *Arabidopsis* fruit formation.

Material and methods

Screening for activation tagged mutants

A stable En-I transposon population of *Arabidopsis thaliana* plants, ecotype Wassilewskija (WS-3) (Marsch-Martinez et al., 2002) was screened for pistil/silique aberrations. Progenies of selected mutants were generated to determine the dominance of the trait and for the identification of the tagged gene.

Southern blot analysis

Genomic DNA from 24 segregating plants and from the parental mutant was isolated (Pereira and Aarts, 1998) and approximately 300ng of DNA was digested with the restriction enzyme *EcoR*I. Equal loading of the DNA was checked by Ethidium bromide staining. DNA was electrophoresed in a 1.0% (w/v) agarose gel in 1x TBE (1.0M Tris, 0.9M boric Acid, 0.01 M EDTA) and blotted onto Hybond N+ membrane (Amersham Pharmacy Biotech) following the normal manufacture's instructions. An 1.3 kb PCR fragment spanning from the 5' end of the *BAR* gene to the 3' end of the right transposon junction (Marsch-Martinez et al., 2002) was labelled by random oligonucleotide priming (Gibco BRL®) and used as a probe for hybridisation.

Identification of the activated gene

To identify the putative activated gene, modified thermal asymmetric interlaced–PCR (TAIL-PCR) (Marsch-Martinez et al., 2002) was used to isolate flanking fragments of the En-I elements. Flanking sequences were compared to the *Arabidopsis* database using BLASTN. The two candidate genes, At5g67050 and At5g67060, were amplified using the following primers, respectively: PRI835: 5'-CATGATGAACAGCGACGATGACGATGAAC-3', PRI836: 5'-CGCTAACCGGTTGGTATGAAAATTAC-3', PRI837: 5'-CTATGGATTCTGACATAATGAAC-3', PRI838: 5'-CATCATCATCTAAGAATCTGTGCATTGCC-3'.

Northern blot analysis was used to examine the expression patterns of the genes. Total RNA was isolated from rosette leaves (RL), cauline leaves (CL), closed flowers (top inflorescence) (CF) and open flowers (OF), from wild type *Arabidopsis* plants, ecotype WS-3. Each RNA sample (5µg) was denatured with 1.5M glyoxal, fractionated on 1.4% agarose gel (w/v) and blotted onto a Hybond N+ membrane (Amersham Pharmacy Biotech). The two genes described above were use as probes for hybridisation. The probes were labelled using random oligonucleotide priming (Gibco BRL®) and the blots were hybridised and washed as described by Angenent et al. (1992). Equal loading of the gel was confirmed by Ethidium bromide staining.

Constructs

To confirm that the cloned flanking fragment contains the open reading frame (ORF) of the activated gene, the ORF of the *NDL1* gene (At5g67060) was cloned using GATEWAY™ cloning technology (Gibco BRL®). Approximately 0.2 ug of total RNA from closed flowers was used as template for the RT-PCR reaction following the manufacturer's instructions. A full length *NDL1* cDNA fragment was obtained by PFU proofreading polymerase (Stratagene) using gene specific primers PRI837 and PRI838. Terminal "A" ends were introduced with 1.5 units Super Taq polymerase (Stratagene) and incubated for 30' at 72 º C followed by cloning in pGEMT-Easy® (Promega). After cloning in pGEMT-Easy®, a *NDL1* cDNA fragment with GATEWAY™ sites was obtained by PCR using the following primers: PRO058: 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTATGGATTCTGACATAATGAACATGATG-3' and PRO059: 5'-GGGGACCACTTTGTACAAGAAAGCTGGGTCATCATCATCTAAGAATCTGTGCATTGCC-3'. The gateway attB1 and attB2 sites, respectively, are underlined.

Subsequently, the fragment was cloned into pDONR207 vector (Gibco BRL®) and recombined with the binary vector pGD625, designed pARC081. Vector pGD625 was generated by cloning the *CaMV35S* expression cassette from pGD120 (Immink et al., 2002), as an *AscI/PacI* fragment, (containing the GATEWAY™ cassette reading frame A, cloned in the MCS in *Xba*I, blunt-ended with klenow polymerase), into pBINPLUS (van Engelen et al., 1995). Seeds were surface sterilized (http://plantpath.wisc.edu/~afb/vapster.html) and sowed on MS medium containing Kanamycin at 50 $mg.L^{-1}$. Resistant plants were transferred to the greenhouse.

For the *pNDL1::GUS* construct, the putative 2.0 kb *NDL1* promoter fragment upstream of the ATG was amplified from genomic DNA of ecotype Columbia, using TAQ plus precision polymerase enzyme (Stratagene) and the following primers containing the GATEWAY™ sites (underlined) upstream of the ATG start: PRO093: 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTCATGGGGAAGAACCATCTAAGGAGAAAATCAG -3' and PRO094: 5'-GGGGACCACTTTGTACAAGAAAGCTGGGTAGAGAAAGATATGGAGAAGCTGATAAAATGG -3'. The fragment was cloned into pDONR207 vector (Gibco BRL®) and recombined with the binary vector pBGWFS7, resulting in pARC 200. For selection, seeds were vapor phase surface-sterilized (http://plantpath.wisc.edu/~afb/vapster.html) and grown directly on soil in the greenhouse. Seven days after germination (DAG) resistant plants were selected by spraying twice with 150 mg L^{-1} Finale®, with an interval of 2 days between each spraying.

Plant transformations

Constructs were transformed into *Arabidospis thaliana* ecotype Col-0 using *Agrobacterium tumenfaciens* strain GV3101 and the floral dip method (Clough and Bent, 1998), unless otherwise described.

***ndl1* knockout mutant**

The *ndl1* mutant was obtained from the GABI-kat facility (Li et al., 2003). The putative knockout mutant was confirmed via segregation analysis and by PCR using the gene specific primer PRI837 and the T-DNA vector left border, primer PR0140 (5'-CCCATTTGGACGTGAATGTAGACAC-3'). The primers PRI837, PRI838, and PRO140 were used to identify homozygous plants. The insertion position was confirmed by sequencing the left flanking sequence of the T-DNA vector (BigDye^{TM} sequencing kit, Applied Biosystems) using primer PRO141 (5'-ATATTGACCATCATACTCATTGC-3').

***In situ* RNA hybridisation**

In situ hybridisations were performed as described by Cañas et al. (1994). Gene specific primers PRO283 (5'-ATGGATTCTGACATAATGAAC-3') and PRO284 (5'-TAATACGACTCACTATAGGGAGAGATAGAAGAAGGGTTAG-3') were used to amplify a 200 bp fragment located at the 5' end of the gene At5g67060. Primer PRO284 contains a T7 RNA polymerase promoter site. Digoxigenin-labeled RNA probes were synthesized by T7 polymerase-driven *in vitro* transcription.

Plant Lines

***DR5::GUS* line**

The line *DR5::GUS* was kindly obtained from Ben Scheres, Utrecht University, Utrecht, The Netherlands and described by Ulmasov et al. (1997).

pFBP1::GUS

The *pFBP1::GUS* construct was reported by Angenent et al. (1993). The construct was introduced into *Arabidopsis thaliana* plants, ecotype Col-0, by Agrobacterium-mediated transformation by floral dipping (Clough and Bent, 1998).

Results

***needle1-D*, a gain-of-function mutant with strong defects in carpel development**

Screening a transposon-based activation tagging population (Marsch-Martinez et al., 2002) for morphological mutants affected in pistil development a putative activation mutant, designated *needle1-D* (*ndl1-D*), was identified. The *ndl1-D* mutant has a late flowering phenotype characterised by abnormal carpel development (Figure 1). Instead of

a normal pistil, a thin needle-like structure is formed which is topped by stigmatic tissue (Figure 1B). The other floral organs appear normal, although occasionally extra petals are formed. Plants are female sterile, due to abnormal pistil development; however, no defects were observed in male fertility. Inflorescences terminate in a pin-like structure (Figure 1C) and, occasionally, this structure curves at the apex and produces carpelloid tissues (Figure 1D). Most likely due to the fact that *ndl1-D* is sterile, there is a lack of apical dominance (Figure 1E) and the mutant plant exhibits a bushy-like appearance, with new stems arising from the axils of the cauline and rosette leaves (Figures 1E and F). No visible phenotype could be seen at the seedling and rosette stages.

Particularly, the early arising flowers are less severe than flowers that develop at later stages of plant development. In these flowers with a weak phenotype, carpel formation is altered at the base of the gynoecium (Figure 1G), whereas the distal part develops a morphologically normal pistil harboring ovules. The ovules are fertile and produce viable seeds (Figure 1H).

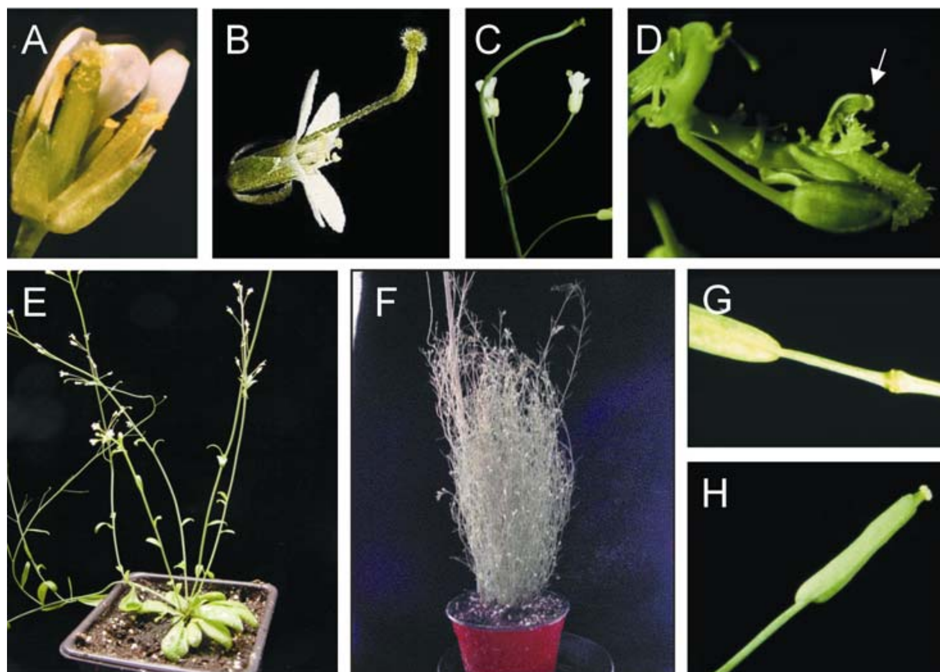

Figure 1. Phenotype of the *ndl1-D* mutant plant. **(A)** Wild type Col-0 flower **(B)** *ndl1-D* flower with the needle-like structure instead of a normal pistil **(C)** Inflorescence of the *ndl1-D* plant showing the pin-like termination **(D)** Terminated inflorescence, that occasionally curves at the apex and produces carpelloid tissues (arrow). **(E)** *ndl1-D* adult plant showing a lack of apical dominance with new stems arising from the axils of the cauline and rosette leaves. **(F)** A bushy adult plant. **(G, H)** Siliques with a weak *ndl1-D* phenotype. At distal part a normal silique is formed containing seeds, while there is no valve formation at the basal part. ***Colour picture, see Appendix.***

***NDL1* encodes a transcription factor belonging to the *Arabidopsis* basic/HELIX-LOOP-HELIX (AtbHLH) family.**

To identify the responsible gene causing the *ndl1-D* overexpression phenotype, the original mutant was backcrossed with its wild type, ecotype WS-3. Because *ndl1-D* is female sterile, a *ndl1-D* plant was used as a male parent. A backcross (BC1) generation was generated and an approximate 1:1 ratio was observed, demonstrating that this is a dominant gain-of-function mutation. To confirm whether this mutation is due to a single copy, Southern blot hybridisation analysis was performed. WS-3 and the parental mutant plant were used as controls. This analysis revealed that a single copy of the activation I element (AIE) was present which cosegregated with the mutant *ndl1-D* mutant phenotype (data not shown).

TAIL-PCR was used to amplify the DNA flanking sequence of the insert (Liu et al., 1995; Liu and Whittier, 1995; Tsugeki et al., 1996). Comparing the isolated flanking sequence with the *Arabidopsis* genome sequence revealed that the insertion was located at chromosome 5 between two genes. The distance between the insert and both genes was approximately 4 kb. Gene At5g67050, encoding a putative lipase-class 3 protein, is upstream and gene At5g67060, which contains a Helix-Loop-Helix (HLH) motif protein, is downstream of the insertion (Figure 2A).

Northern blots were performed with samples from rosette leaves (RL), stems (S), closed flower buds (top of inflorescence) (CF) and open flowers (OF), from *ndl1-D* and wild type plants, WS-3 and Col-0. There is a clear increase in expression of the HLH gene, whereas there is no change in expression of the other gene (Figure 2B). This shows that overexpression of At5G67060, now referred to as *NDL1*, is most likely responsible for the phenotype of the *ndl1-D* mutant. Recently, the *Arabidopsis* bHLH protein family was characterized (Toledo-Ortiz et al., 2003). *NDL1* (AtbHLH88) belongs to a sub-clade, within the subfamily 19. This clade contains five proteins of unknown function that share high homology within the HLH domain (Figure 3). One member, AtbHLH37, has the same bHLH domain sequence as *NDL1* (Toledo-Ortiz et al., 2003).

Overexpression of *NDL1* by the *35S* promoter recapitulates the *ndl1-D* phenotype

To confirm that the overexpression of *NDL1* by the 4x*35S* enhancers is the cause of the *ndl1-D* phenotype, the predicted full-length *NDL1* cDNA under the control of the *35S* promoter was introduced into *Arabidopsis* plants. Out of 129 resistant plants, only 5 plants showed a mild *ndl1-D-like* phenotype (Figure 4A). Like the severe *ndl1-D* activation tagging phenotype, lateral branching is more prominent in these overexpression lines compared to wild type and aberrant pistils and siliques develop, which are reminiscent with mild *ndl1-D* activation mutants (Figure 4B). At other positions in the flower (Figure 4C,

D) in the *35S::NDL1* transgenic plants, sepals are replaced by organs with carpelloid features.

This difference in severity between the activation tagging line and the overexpression line might be due to the genetic background used in the transformation. The activation tagging line has a WS-3 background, whereas the overexpression line is in Col-0.

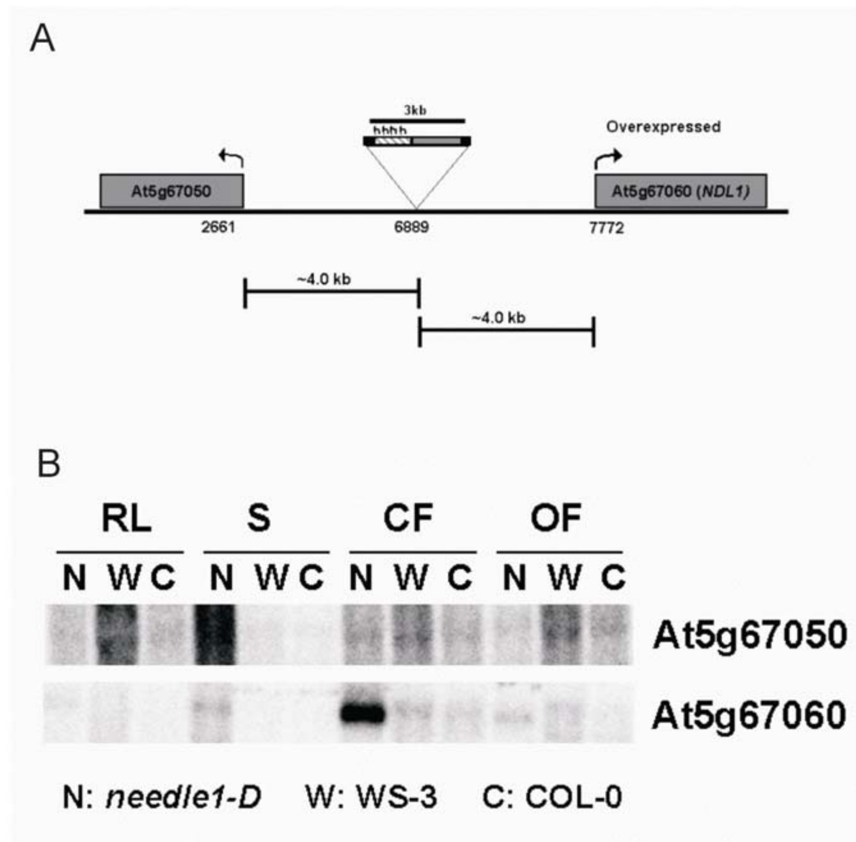

Figure 2. (A) Schematic representation of the insertion position of the activation I element (AIE) in the *ndl1-D* genome. The insertion was located between two genes at chromosome 5. The distances of the genes to the 4x*35S* enhancer elements in the AIE are represented by black lines with the distance indicated in kilo basepairs (kb). Gray boxes depict the two genes and the arrows represent the direction of transcription. Numbers beneath the gray boxes show where the ATG start codon is positioned and the number beneath the AIE represent the insertion point in the genome. The AIE element is about 3.0 kb in length and is composed of the 4x*35S* enhancers (dashed box) and the *BAR* resistant gene (filled gray box). The black boxes are the left and the right junctions of the AIE element. **(B)** Northen blots showing the expression of the two genes present in the vicinity of the AIE. Upper blot was hybridised with the upstream gene (At5g67050) and lower blot with the

downstream gene (At5g67060). Samples were collected from rosette leaves (RL), stems (S), closed flower buds (top of inflorescence) (CF) and open flowers (OF) from *ndl1-D* (N) plants and wild type plants WS-3 (W) and Col-0 (C).

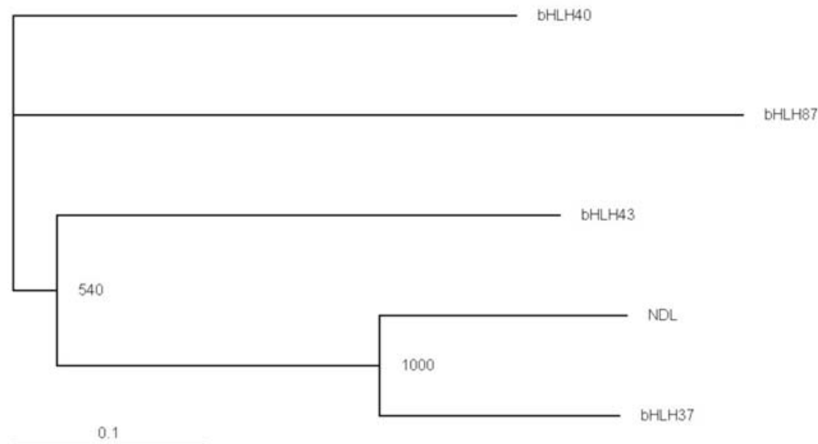

Figure 3. Phylogenetic tree of the group VIIIb of the basic/Helix-Loop-Helix transcription factor family in *Arabidopsis*. The tree was generated by ClustalX program using Phylip distance matrix with 1.000 bootstrap and the graphic representation was obtained by TreeView software, version 1.6.6

Whorl 4 has no identity change in a *ndl1-D* mutant

The needle-like structure of the pistil present in the *ndl1-D* mutant can be compared to a filament-like organ, reminiscent with the filament of stamens. To understand more about the morphological changes that may have occurred in the *ndl1-D* mutant, a marker for whorls 2 and 3 (petals and stamens) was used. A petunia marker, *FLORAL BINDING PROTEIN1* (*FBP1*), specific for petal and stamen identity was used, which should mark possible changes in identity from pistil to stamen filament (Angenent et al., 1993). An *Arabidospsis* line, containing a *pFBP1::GUS* construct was crossed with the activation tagging line *ndl1-D*. Transgenic F1 plants showing a *ndl1-D* phenotype and expressing GUS were analysed. F1 plants that did not show the *ndl1-D* phenotype were used as a control. In wild type *Arabidopsis* flowers, *pFBP1::GUS* is expressed in petals and filaments (Angenent et al., 1993). In *ndl1-D* plants, there was no difference in the temporal or spatial expression of the *FBP1* promoter compared to wild type plants. Based on these GUS results it was concluded that the structure observed in the *ndl1-D* mutant has no link to stamen filament identity (results not shown).

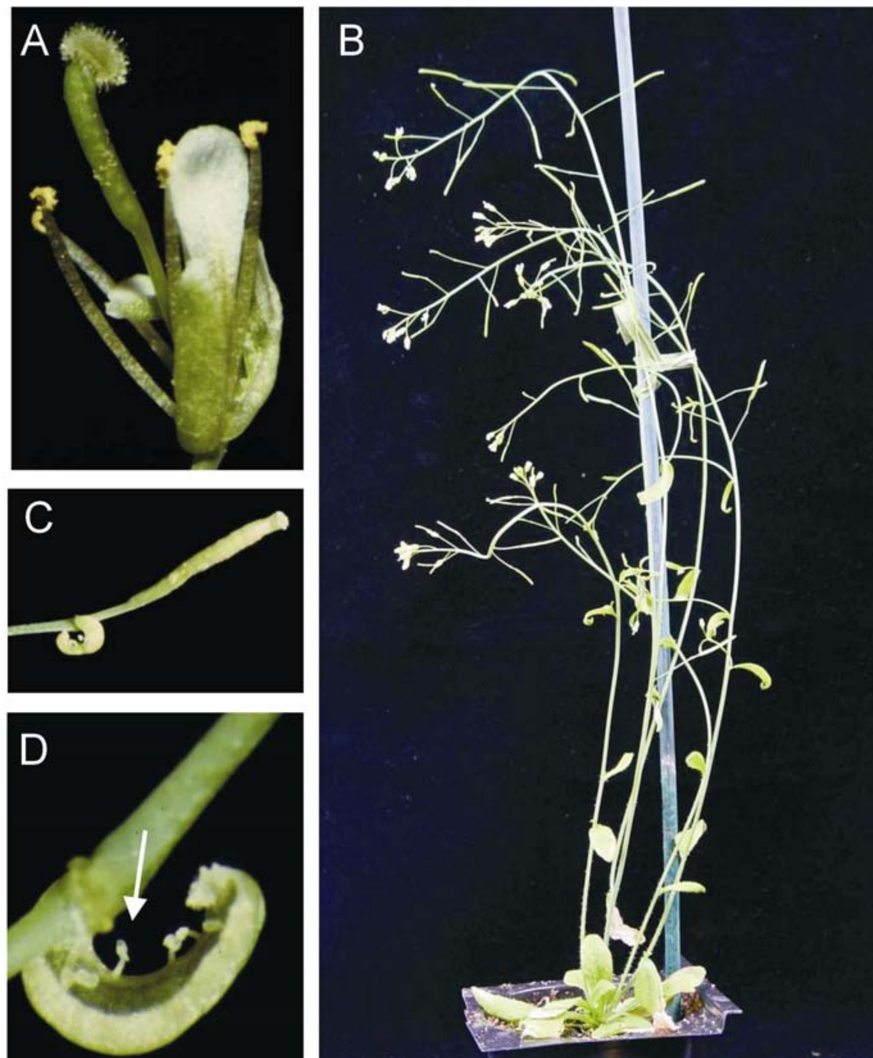

Figure 4. Phenotypes of the *35S::NDL1* plants. **(A)** Single flower of the *35S::NDL1* transgenic line showing partial valve formation just at the distal part of the pistil. **(B)** An adult *35S::NDL1* plant with extra stems arising from rosette leaves leading to more prominent lateral branching. **(C)** Occasionally, sepals are replaced by organs with carpelloid features. **(D)** Close-up of a carpelloid organ in the first floral whorl. An arrow points to an ovule-like structure. ***Colour picture, see Appendix.***

ndl1-D **mutant and auxin accumulation**

The *ndl1-D* phenotype is similar to the previously described loss-of-function *pinoid* (*pid*) and *ettin* (*ett*) mutants and also shows characteristics of the *pin-formed* (*pin*) mutant (Bennett et al., 1995; Sessions et al., 1997; Christensen et al., 2000; Benjamins et al., 2001). *pin* mutants develop a pin-like inflorescence and, occasionally, flowers are formed

showing less sepals, more petals, no stamens and an abnormal pistil (Okada et al., 1991). The phenotype of *pid* is similar to *pin*, but is less severe. Inflorescences end in a pin-like structure and bear only a few aberrant flowers, generally containing few or no sepals, more petals and a trumpet shaped pistil (Benjamins et al., 2001). The *ettin* mutant displays tissue patterning defects in carpels and is postulated to be involved in prepatterning apical and basal boundaries in the gynoecium primordium (Sessions and Zambryski, 1995). Besides the phenotypic similarities, these three mutants have been described to be involved in the auxin signalling pathway (Bennett et al., 1995; Nemhauser et al., 1998; Christensen et al., 2000; Nemhauser et al., 2000; Benjamins et al., 2001).

A reporter gene that is induced in response to auxin was used to analyse the relation of *ndl1-D* and the auxin pathway. *ndl1-D* was crossed to a plant carrying the *DR5::GUS* reporter, which contains a multimerized synthetic auxin response element that is bound by ARF (auxin response factor) transcription factors (Ulmasov et al., 1999b). Because *DR5::GUS* expression has been studied mainly in roots, GUS expression in roots was used as a control. Depicted in figure 5A, *DR5::GUS* wild type expression is concentrated in root tips. In the aerial portion of wild type plants, GUS expression is present only in anthers, at the anthesis stage (Figure 5C). In *ndl1-D* plants, the reporter gene is expressed in the needle-like structure of the gynoecium, particularly at the apex. Expression also appears stronger in anthers (Figure 5D). These results indicated that auxin levels are elevated in *ndl1-D*, mainly in the altered gynoecium.

***ndl1* loss-of-function**

ndl1-D is a dominant gain-of-function mutant, which most likely acts in the auxin pathway. To better understand the *NDL1* function, a loss-of-function mutant was isolated. The GABI-Kat population (Li et al., 2003) was screened and two insertions were found in the *NDL1* gene. Due to an insertion in the HLH domain, line GABI-kat # 297B10 was chosen for further characterisation. The T-DNA insertion is located at position 541 bp in the gene and lies within the HLH domain. The presence of the insertion was confirmed by sequencing the T-DNA flanking sequences. T2 seeds were sown and a phenotypic screen was performed. Homozygous plants were identified by PCR using T-DNA and gene specific primers. Out of 48 plants, 11 were classified as being homozygous for the presence of the T-DNA insertion. Unfortunately, no clear phenotype could be seen in those plants, under normal greenhouse conditions. This suggests that *NDL1* is most likely redundant with other members of the HLH family and therefore, the generation of double mutants with a few candidate paralogues is in progress.

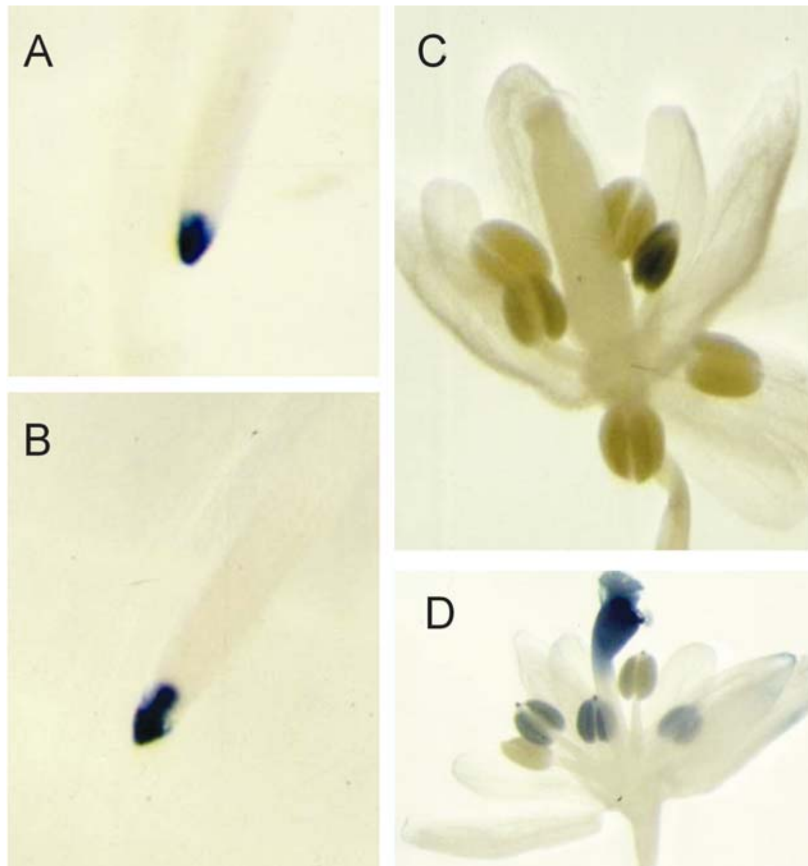

Figure 5. Macroscopic analysis of the *DR5::GUS* expressing plants. **(A)** Wild type root tip with *DR5::GUS* expression at the tip. **(B)** *ndl-1D* mutant plant expressing *DR5::GUS* in the root tip. **(C)** Wild type flower containing *DR5::GUS*. **(D)** GUS expression in a *ndl1-D* flower. GUS activity is high in anthers and at the top of the needle-like structure of the gynoecium. ***Colour picture, see Appendix.***

***NDL1* expression**

The *NDL1* expression pattern was examined using a *NDL1* promoter *GUS* fusion. A 2.0 kb region upstream of the predicted ATG start codon was cloned, fused to the β-glucoronidase gene (*GUS*), and introduced to COL-0 wild type plants. Rosette leaves, cauline leaves, young lateral inflorescences, inflorescences with flower buds and siliques up to stage 17 (Ferrandiz et al., 1999) were collected from T1 plants and analysed for GUS activity. Out of 24 plants, only two plants did not show any GUS expression. Among the others, 12 showed the expression depicted in figure 6. There is no expression in rosette leaves or in cauline leaves. In the flower (Figure 6A-C), GUS expression is absent in the first three floral whorls, sepals, petals and stamens, but is restricted to the apex of the pistil.

NDL1 expression is seen just below the stigma, extending downwards through the septum. Sometimes, expression appears throughout the entire pistil (Figure 6B). In older pistils, expression is still observed in the stylar zone and septum (Figure 6C).

The activity of the *NDL1* promoter is concentrated in gynoecium tissues. To determine whether the stage-specific promoter GUS expression pattern correlates with *NDL1* transcripts and to gain more insight in *NDL1* expression, *in situ* RNA hybridisations were performed in wild type plants. *NDL1* transcripts are detectable in the inflorescence meristems and also in emerging flowers. In the inflorescence meristems, the expression is limited to the apex of the meristem (Figure 6D). At later floral developmental stages (Figure 6E), *NDL1* is strongly expressed in the carpel primordia and is also detected in stamens. At a later stage, *NDL1* expression is observed at the region of carpel fusion, in cells lining the central invagination and the distal part of the developing gynoecium (Figure 6F). Furthermore, expression is present in the locules of the anthers, where tapetum and microspores develop.

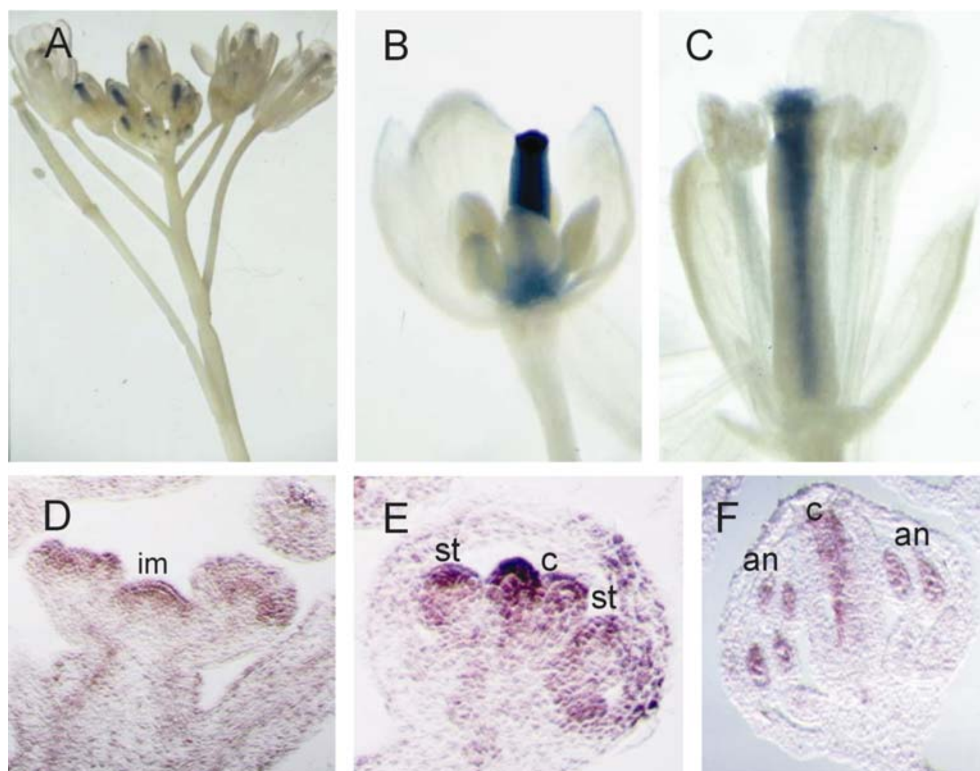

Figure 6. Expression analysis of *NDL1*. **(A-C)** *pNDL1::GUS* activity and **(D-F)** *in situ* RNA hybridisation analysis on longitudinal sections and hybridised with DIG labeled antisense probe. **(A)** Inflorescence of the *pNDL1::GUS* transgenic line. The GUS staining is seen in the gynoecium of the flowers. **(B)** Young floral bud showing GUS activity in the entire pistil **(C)**. At a later stage, expression is restricted to the distal zone and septum. **(D)**

In the inflorescence meristems (im) *NDL1* is weakly expressed at the apex of the meristem and in floral meristems. **(E)** Floral bud showing mRNA accumulation in floral organ primordia. Expression is high in carpel (c) and stamen (st) primordia. **(F)** Flower bud at later developmental stage. *NDL1* is expressed at the region of carpel (c) fusion, in cells lining the central invagination and also in the anther locules (an). ***Colour picture, see Appendix.***

Discussion

NDL1 is involved in polar auxin transport

NDL encodes a transcription factor belonging to the recently described *Arabidopsis* bHLH transcription factor superfamily, which has not been well characterised in plants so far (Toledo-Ortiz et al., 2003). Transcription factors are known to be important regulatory proteins involved in all plant development processes (Riechmann and Ratcliffe, 2000; Parenicova et al., 2003). HLH proteins control gene expression by binding to specific DNA sequence motifs as homo or heterodimer combinations (Toledo-Ortiz et al., 2003).
The *ndl1-D* mutant is characterised by abnormal carpel development. Similar defects in carpel development are also observed for the loss-of-function mutants, *pin*, *pid* and *ett*. In addition to an effect on floral phenotype, these genes play a role in auxin-related processes. Many genes have been reported that when overexpressed, influence directly or indirectly the auxin pathway, either in the signaling pathway or in transport (Benjamins et al., 2001; Zhao et al., 2001; Tian et al., 2002).

Likewise, here we demonstrated that the *ndl1-D* mutant has also an affect on auxin levels. When an auxin-responsive reporter gene construct, *DR5::GUS* was introduced in *ndl1-D* mutant plants, higher levels of GUS activity, which is correlated with levels of auxin, could be detected in the needle-like structure. In contrast, no change in auxin levels in the inflorescence were detected in *pid* and *pin* mutants (Bennett et al., 1995; Benjamins et al., 2001). The authors postulated that auxin levels remain constant due to an indirect effect of the absence of developing carpels and ovules, known sites of auxin synthesis (Lomax et al., 1995). In the *ndl1-D* plants, there is no silique formation either, but the altered pistil seems to be sufficient for the accumulation of auxin.

Auxin plays a crucial role throughout the life of a plant from early embryogenesis to fruit development through cell division, elongation and differentiation (Gälweiler et al., 1998; Sabatini et al., 1999; Nemhauser et al., 2000; Hamann, 2001; Stieger et al., 2002; Takada and Tasaka, 2002); (Chen, 2001). There is clear evidence that polar auxin transport is the main factor of the auxin-regulated pathway. The main polar auxin transporter protein is PIN. Reinhardt et al. (2000) treated *pin* plants by microapplication of the natural auxin indole-3-acetic acid (IAA) to the apex, restoring leaf and flower formation. Thus,

demonstrating that the mutation in the *PIN* gene blocks polar auxin transport and subsequently, leaf and organ morphogenesis. The suppression of either *PIN* or *PID* results in an inhibition of auxin transport. This effect can be mimicked in wild type plants by spraying them with auxin polar transport inhibitors (Nemhauser et al., 2000), such as 1-N-naphtylphthalamic acid (NPA) (Okada et al., 1991). Treated plants stopped producing normal flowers and instead, the pin-like structure appears. This phenotype is strongly similar to both *pin* and *pid* mutants, indicating that these proteins are indeed related to auxin transport.

The observed similarity between *ndl1-D* and *pid* knockout mutants temps speculation that the gain-of-function *ndl1-D* mutant is a result of *PID* pathway blockage. *PID* and *NDL1* have overlapping expression patterns suggesting that both proteins are present in the same tissue (Christensen et al., 2000; Benjamins et al., 2001). This allows the speculation that they might be involved in the same pathway in an antagonistic way. When there is an increase in *NDL1* product, it may negatively regulate *PID* expression, resulting in the *ndl1-D* phenotype. In such a situation, NDL1 could directly bind to the *PID* promoter as a suppressor. A similar relationship has been reported for *SPT* and *ETT*. It has been proposed that ETT binds to auxin response elements (ARE) in the *SPT* promoter, subsequently blocking SPT transcription in the developing gynoecium (Heisler et al., 2001). Future experiments should reveal whether this model of *PID* regulation by NDL1 is true.

***NDL1* is involved in carpel development**

Based on the expression pattern, *NDL1* is most likely involved in carpel development. It is highly expressed in the carpel primordia and at later developmental stages at the distal part of the pistil. However, in the loss-of-function *ndl1* mutant, no function can be attributed to this gene due to a lack of mutant phenotype. Two other bHLH proteins recently cloned *ALCATRAZ (ALC)* (Rajani and Sundaresan, 2001) and *SPATULA (SPT)* (Heisler et al., 2001) are involved in fruit development. These AtbHLH genes (Toledo-Ortiz et al., 2003), as well as *ETTIN (ETT)* (Sessions et al., 1997), have been shown to play important roles in gynoecium development. Mutations in these genes lead primarily to abnormal gynoecium development (Sessions et al., 1997).

The results of the *NDL1* expression patterns are in line with other data that reveals that *NDL1* expression is restricted to pistil/silique development (de Folter, unpublished results). Heim et al., (2003) also demonstrated that *NDL1* and its closely related homolog, AtbHLH87, are only expressed in floral and fruit tissues. This restricted expression of *NDL1* in the gynoecium and developing silique suggests that *NDL1* functions in female organ development. Furthermore, the *ndl1-D* mutant specifically affects gynoecium

development, although this is an overexpression situation and may not reflect the biological activity of the protein.

Auxin transport or perception has been postulated to play a major role in gynoecium development (Bennett et al., 1995; Nemhauser et al., 2000). In this model, auxin is produced distally and is actively transported basipetally, forming a concentration gradient. The identities of gynoecium tissues, such as stigma, style, valve and gynophore, are promoted by high, medium and low auxin concentration, respectively, demonstrating the fate-determining role of auxin (Golz and Hudson, 2002). In the mild *ndl1-D* mutants, there is clearly an accumulation of auxin in the remaining carpel tissue at the top of the needle-like structure, with no basipetal translocation seen in the gynoecium. This effect is also seen in *ett* mutants. In the most severe *ett* mutant, the complete valve is absent forming a style-like structure. Application of polar auxin transport inhibitors to a weak *ett* allele causes a more severe mutant phenotype, implying that ETT perceives the auxin concentration gradient (Nemhauser et al., 2000). This auxin gradient is important to delimit cell boundaries between gynoecium tissues: the apical boundaries between the style and valve initials and the basal boundary between the valve and receptacle (Golz and Hudson, 2002). When the basipetal gradient between the apical boundaries is disturbed, the boundaries in the pistil are shifted. Changes in these auxin gradients are very likely to happen in *ndl1-D*, giving rise to aberrant boundaries. When the required threshold of auxin is not reached in the ovary, the strong *ndl1-D* phenotype appears, with stigmatic tissue on top of a pedicel-like structure. In the mild mutant, the top of the gynoecium develops normally with a style, stigma and a partial ovary, whereas the basal part of the ovary is replaced by a short pedicel-like structure. In this case, the auxin threshold was apparently reached to overcome a minimum concentration required for specifying the apical boundary.

Conclusion

Here, information about the function of a so far uncharacterized member of the bHLH transcription factor family is given. Knockout mutant analysis revealed that *NDL1* is most likely redundant with other closely related members of this family. Double or even triple mutant studies should shed light on the function of these genes in plant development. Furthermore, the relationship with two other bHLH members involved in pistil development, *SPT* and *ALC*, has to be elucidated by genetic and molecular analyses. Moreover, *NDL1* is most likely involved in pistil development via the auxin-signaling pathway acting antagonistically to other regulators such as *PIN*, *PID* and *ETT*. The exact role and position of *NDL1* in the auxin-pathway controlling gynoecium development, however, remains unresolved and requires further investigations.

Acknowledgments

The authors thank Andy Pereira's group, Rafaella Greco, Asaph Aharoni for useful discussions. Thanks to all members of Gerco Angenent's lab for all contributions during this work, especially Faye Rosin for English corrections. Maarten Koornneef and Mark Aarts are acknowledged for fruitful ideas and discussions. This work has been financially supported by CAPES-Brazil (BEX 1519/98-0).

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CHAPTER 7

Overexpression of the *Arabidopsis TWISTED1* gene affects plant architecture

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Abstract

Screening an activation tagging population, the gain-of-function mutant *twisted1-D* was obtained. In this mutant, all organs become twisted with a severe phenotype observed in the siliques. The *TWT1* gene was cloned and encodes a putative transmembrane protein of unknown function. Expression analysis revealed that *TWT1* is expressed in the vascular system of the entire plant and in trichomes. A knockout mutant of *TWT1* did not show any phenotype suggesting redundancy with other *Arabidopsis* homologues. The gain-of-function phenotype combined with the expression analysis suggest that *TWT1* is involved in vascular tissue development. Furthermore, a link can be made between the twisted phenotype and auxin transport or perception.

Introduction

The trafficking of molecules along the plant body is vital for the growth of all organs. This intracellular and long distance communication takes mainly place within the vascular system of the plant. Plants use the vascular system to connect distant organs, like the roots with the flowers and it serves as a major driving force behind plant development. This vascular system, which is a non-circulatory network, is the route taken to efficiently exchange nutrients and signalling molecules. There are two important components of the vascular system: xylem and phloem. The xylem acts to transport water and minerals absorbed by the roots to aerial parts of the plant, and the phloem transports photoassimilates from the leaves, where they are produced, to growing tissues (Haywood et al., 2002). The formation of the vascular system during organ development has been used as a model to study pattern formation and tissue differentiation in plants (Koizumi et al., 2000). Basic processes such as cell division, cell growth and differentiation are studied in detail, although the genetic control of vascular tissue identity is not well understood (Bonke et al., 2003). Phytohormones play also an essential role in vascular development (Aloni, 1987). One of the main phytohormones, auxin, has been proposed to influence several aspects of plant development, including differentiation of vascular tissue (Lomax et al., 1995). The *Arabidopsis lopped1* (*lop1*) mutant is affected in the basipetal auxin transport, giving rise to abnormalities in vascular development, lateral root initiation and patterns of cell expansion (Carland and McHale, 1996). In addition, as a consequence of the primary defect in auxin transport all organs of the *lop1* mutant are twisted (Carland and McHale, 1996).

Before the phloem and xylem cells differentiate in a specific manner, their identity has to establish (Berleth et al., 2000). Recently, it has been reported that *ALTERED PHLOEM DEVELOPMENT* (*APL*) is important to define phloem-xylem identity. *APL* has a dual role in both promoting phloem differentiation and in repressing xylem fate during vascular development. *apl* mutant seedlings display determinate root growth and arrested shoot development, which is caused by an aberrant vascular system (Bonke et al., 2003). A mutation in *CONTINOUS VASCULAR RING* (*COV1*), a transmembrane protein, leads to a dramatic increase in vascular tissue formation in the stem. The interfascular region, which normally separates the vascular bundles, is replaced by vascular tissue, resulting in a ring-like pattern of xylem and phloem (Parker et al., 2003).

The mis-specification of cell fate is also observed in another mutant, *tornado1-1* (*trn1-1*) (Cnops et al., 2000), which displays twisted roots and twisting of other organs. *TRN1* has been proposed to maintain the spatial organisation of the epidermal initial cells surrounding the central cells in the root. The phenotype of the rest of the plant is not described in detail (Cnops et al., 2000).

From an *Arabidopsis* activation transposon tagging population, a mutant with twisted organs was isolated and named *twisted1-D* (*twt1-D*). Molecular identification showed that the mutant phenotype is due to the overexpression of the endogenous *TWT1* gene by the presence of the tetramer of the Cauliflower Mosaic Virus (*CaMV*) *35S* enhancer (4x*35S*). Overexpression of a genomic clone of *TWT1* under the control of the *35S* promoter resulted in a similar phenotype, demonstrating that the phenotype was due to the upregulation of *TWT1*. *TWT1* encodes for a putative transmembrane protein with unknown function. Based on the overexpression phenotype combined with expression pattern results, it is postulated that *TWT1* is involved in vascular tissue development, either in the perception or in the transport of signalling molecules through the membranes.

Material and methods

Screening for the activation line mutant

The stable En-I transposon population of *Arabidopsis thaliana* plants, ecotype Wassilewskija (WS-3) (Marsch-Martinez et al., 2002) was visually screened for pistil/silique aberrations under normal greenhouse conditions. From the selected *twt1-D* mutant the F1 segregating generation was grown to examine the dominance of the mutation and copy number of the En-I element.

Southern blot analysis

Genomic DNA from 24 segregating plants and the parental mutant was isolated (Pereira and Aarts, 1998) and approximately 300ng of DNA was digested with restriction enzyme *EcoR*I. Equal loading of the DNA was checked by Ethidium bromide staining. DNA was eletrophoresed in a 1.0% (w/v) agarose gel in 1x TBE (1.0M Tris, 0.9M boric Acid, 0.01 M EDTA) blotted onto a Hybond N+ membrane (Amersham Pharmacy Biotech) following the normal manufacture's instructions. A 1.3 kb PCR fragment amplified from the 5’ end of the *BAR* gene and from the 3’ end of the right transposon junction, was labelled by random oligonucleotide priming (Gibco BRL®) and used as a probe (Marsch-Martinez et al., 2002).

Identification of the activated gene

To identify the putative activated gene, genomic DNA was used to isolate flanking fragments of the En-I elements by modified thermal asymmetric interlaced–PCR (TAIL-PCR) as described previously (Marsch-Martinez et al., 2002). Flanking sequences were compared to the *Arabidopsis* database using BLASTN. The insertion was located in a genomic region between two genes, approximately 8.2 kb downstream of gene At2g32270, and 3.6 kb upstream of gene At2g32280. This distance is predicted from the 4x*35S* enhancers located at the left border of the T-DNA. To amplify the genes the following

primer combinations were used for At2g32270 and At2g32280 respectively: PRO011: 5'-GATTATGAAGACTAAGAACGTGAAAC-3', and PRO012: 5'-AGCTATCAAGCCCATTTAGCC-3', PRO013: 5'-TTGAAAATGACAAAGATAGGAGG-3', and PRO014: 5'-CTTTGTCACTTAGCTTCATCTTTGG -3'. RNA expression was checked by Northern blot analysis. Total RNA from the *twt1-D* and wild type WS-3 was isolated from roots (R), rosette leaves (RL), cauline leaves (CL), closed flowers (top of the inflorescence) (CF), open flowers (OF), stems (ST) and siliques (S) using the Qiagen® RNA isolation mini kit. RNA (5µl) was denatured with 1.5M glyoxal, fractionated on 1.4% agarose gel (w/v) and blotted onto a Hybond N+ membrane (Amersham Pharmacy Biotech). The two genes described above were use as probes for hybridisation. The probes were labelled using random oligonucleotide priming (Gibco BRL®) and blots were hybridised and washed as described by Angenent et al. (1992). Equal loading of RNA was checked by Ethidium bromide staining.

Constructs

For the confirmation of the overexpressed gene, cDNA of the presumed *TWT1* gene (At2g32280) was cloned using GATEWAY™ cloning technology (Gibco BRL®). Approximately 0.2 ug of total RNA from closed flowers was used as template for the RT-PCR reaction following the supplier's instructions. The predicted *TWT1* cDNA fragment was obtained by PFU proofreading polymerase (Stratagene), using the primers PRO062 and PRO063. PRO062: 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTATGACAAAGATAGGAGGTATTCTTGTTTTG -3' and PRO063: 5'-GGGGACCACTTTGTACAAGAAAGCTGGGTCTTTGTCACTTAGCTTCATCTTTGGCC -3'. The GATEWAY™ attB1 and attB2 sites are underlined. Subsequently, the fragment was cloned into pDONR207 vector (Gibco BRL®) and recombined to the binary vector pGD625, now called pARC 083. The pGD625 vector was generated by cloning the *CaMV35S* expression cassette from pGD120 (Immink et al., 2002), as an *Asc*I/*Pac*I fragment, (containing the GATEWAY™ cassette reading frame A, cloned in the MCS in *Xba*I, blunted with klenow polymerase), into pBINPLUS (van Engelen et al., 1995). The construct was then introduced into *Arabidopsis* plants. Surface sterilized seeds (http://plantpath.wisc.edu/~afb/vapster.html) were sown on MS medium containing 50 mg.L^{-1} Kanamycin. Resistant plants were transferred to the greenhouse.

For the construct pARC170, a two-step cloning strategy was used to clone a 6.5 genomic fragment of the *twt1-D* mutant, starting from the *NOS* terminator within the AIE element to 446 bp downstream of the predicted stop codon. This 6.5 kb *twt1-D* genomic fragment was divided into two fragments to facilitate cloning. In all PCR amplification

steps, TAQ plus precision polymerase enzyme (Stratagene) was used. Fragment "A" was amplified using the primers PRO142 and PRO143, resulting in a 4.5 kb fragment spanning the *NOS* terminator to 1.2 kb upstream of the putative ATG. The "B" fragment was amplified with the primers PRO097 and PRO176. This 3.2 kb fragment contains the region 2.0 kb upstream of the ATG to 446 bp upstream of the predicted stop codon. Each fragment was cloned independently into pGEMT-Easy®, digested with the restriction enzymes *SST*II and *BsmB*I and ligated to each other resulting in the full 6.5 kb *twt1-D* genomic sequence in pGEMT-Easy®. Primers PRO142 and PRO176 contain additional GATEWAY™ sequences allowing recombination into the pDONR207 vector (Gibco BRL®), creating pARC169; followed by LR recombination step into the vector pkGW (Karimi et al., 2002), establishing pARC170. The primer sequences with GATEWAY™ sites underlined are the following for PRO142: 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTTATCGCGCGCGGTGTCATCT-3'; PRO143: 5'-GGGGACCACTTTGTACAAGAAAGCTGGGTGTTAATTAACATTTCTTCAAGTG-3'; PRO096: 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTATCCTACATCTCGGCGAGAGCGGGATTGAACA -3' and PRO176: 5'-GGGGACCACTTTGTACAAGAAAGCTGGGTGATCTTCTTCTGGTAGTAAAA-3'.

The construct pARC173 was built from a fragment amplified from *twt1-D* genomic DNA with TAQ plus precision polymerase enzyme (Stratagene), using the primers PRO062 and PRO176. The amplified 1.0 kb includes the region from the ATG start codon to 446 bp downstream of the stop codon and was cloned into pGEMT-Easy®, recombined into pDONR207, followed by recombination into the final vector pGD625, designated pARC173. The sequence for primer PRO062 with the GATEWAY™ sequences underlined is 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTATGACAAAGATAGGAGGTATTCTTGTTTTG-3'. Primer PRO176 is described above.

The putative 2.0 kb *TWT1* promoter fragment was amplified from genomic DNA of ecotype Columbia (Col-0), using TAQ plus precision polymerase enzyme (Stratagene). The following primers containing the GATEWAY™ sites (underlined) were used: PRO097: 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTATCCTACATCTCGGCGAGAGCGGGATTGAACA-3' and PRO098: 5'-GGGGACCACTTTGTACAAGAAAGCTGGGTTTTCAAATCTCTTAACTTAGTAGGATTTAGT-3'. The fragment is directly upstream of the ATG start codon. The fragment was cloned into pDONR207 vector (Gibco BRL®) and recombined into the binary vector pBGWFS7, designated as pARC 202.

For selection, seeds were sown directly onto soil after vapor phase surface-sterilized (http://plantpath.wisc.edu/~afb/vapster.html) Resistant plants were selected after spraying twice with 100 mg L^{-1} Finale®, 7 days after germination (DAG).

Plant transformations

All constructs were transformed into *Agrobacterium tumenfaciens* strain GV3101 and introduced into *Arabidospis thaliana*, ecotype Col-0 by the floral dip method (Clough and Bent, 1998), unless otherwise described.

RACE

Rapid amplification of cDNA ends (RACE) was performed to amplify the 5’ cDNA end of *TWT1*, using SMART™ RACE cDNA amplification kit from CLONTECH. The following primers were used: the gene specific primer (GSP) PRO180: 5’-AGACAGCCTCCGACCAAGTTAAG-3’, and the nested primer on GSP (nGSP) PRO181 5’-CTGGCTCGGCTCTCTGCACACAA-3’. The annealing temperature used was 58º C for both amplification reactions (GSP and nGSP) with 35 cycles. Selected bands were excised from gel and purified by GFX™ DNA gel purification column kit (Amersham Biosciences).

***twt1* knockout mutant**

The *twt1* mutants were obtained from the NASC stock centre (Nottingham *Arabidopsis* Stock Centre) from SALK population accession numbers N547972 and N523737. The knockout lines for the gene At4g21310 are from the SALK lines, N599815 and N599839. GARLIC lines were obtained directed from Torrey Mesa Research Institute (Syngenta Research and Technology) accession numbers Garlic_893_G06.b.1b.Lb3Fa and Garlic_237_C09.b.1a.Lb3Fa. Putative knockout mutants were confirmed via segregation analysis and by PCR with the gene specific primer PRO014 and T-DNA vector left border primer LB3 for the GARLIC lines and with PRO014 and LBb1 for the SALK lines. To confirm insertion homozygosity, the gene specific primer and the T-DNA vector left border primer were used. The insertion position was confirmed by sequencing the left flanking sequence of the T-DNA vector (BigDye™ sequencing kit, Applied Biosystems) by using T-DNA specific primers described above. The insertions were positioned in the genes as follows: In the *twt1* SALK line N547972, in the third exon, in the line N523737, in the first intron. In the *twt1* Garlic_893_G06.b.1b.Lb3Fa, in the first intron and in the line Garlic_237_C09.b.1a.Lb3Fa, in the second exon. In the At4g21310 SALK line, N599815, in the second exon and in the line N599839, in the first exon.

In situ RNA hybridisation

In situ hybridisations were performed as described by Cañas et al. (1994). Digoxigening-labeled RNA probes were synthesized by T7 polymerase-driven *in vitro* transcription from the PCR fragment amplified with primers PRO287 5'-GTGAAGCACATGAGGCTTTGG-3' and PRO288 5'-TAATACGACTCACTATAGGGCAAGTGAGGACAAGACAAGC-3'. Primer PRO288 contains the T7 polymerase promoter site. The 200 bp amplification product contains the entire second exon of the gene At2g32280.

Databases

The blast programs of the following databases were used: NCBI (National Centre of biotechnology Information - http://www.ncbi.nlm.nih.gov), TAIR (*Arabidopsis* Information Resource - http://www.arabidopsis.org) and TIGR (http://www.tigr.org). For the gene prediction, the gene locus was entered at http://www.tigr.org/tdb/e2k1/ath1/LocusNameSeach.shtml, then at the gene structure link the figure 5 was obtained.

Results

twisted1-D (*twt1-D*), a dominant activation tagging line with a modified plant architecture.

A plant with aberrations in pistil/silique growth was isolated from an activation tagging population generated by a transposon tagging approach using the En-I system (Marsch-Martinez et al., 2002). Because the phenotype of the mutant is characterized by twisting of the organs, the mutant was designated *twisted1-D* (*twt1-D*). Although, the architecture of the entire plant is affected, the most striking alteration is in the silique. In wild type plants the pistil is composed of two fused carpels that are in parallel to one another (Figure 1A). In the *twt1-D* mutant the two fused carpels and the septum exhibit a helical growth in a counterclockwise direction (Figure 1B). Interestingly, no effect on fertility was observed. The mature *twt1-D* siliques are partially shatterproof and contain normal numbers of viable seeds. This twisted growth pattern is observed throughout the entire plant. Whereas cotyledons appear normal, the rosette leaves are twisted. In wild type rosette leaves, the petiole and the blade are flat and positioned horizontally (Figure 1C). In the *twt1-D* mutant the petiole and the blade grow at an angle of 45 degrees and also twist in a counterclockwise direction (Figure 1D). The twisting phenotype is also observed in stems, which affects the radial pattern in the inflorescence (Figure 1F). Furthermore, there is a loss of apical dominance in *twt1-D* plants: lateral branches arise from the rosette and cauline leaves earlier than in wild type plants (Figure 1H). Additionally, *twt1-D* plants have shorter and bushy roots (Figure 1I, J) with a different direction of the root growth

compared to wild type. On vertical agar plates, root growth of wild type plants is slightly deviated to the left, whereas, under the same conditions, *twt1-D* roots do not show any obvious direction of growth (Figure1I).

To analyse the inheritance of this mutant 24 F1 plants were raised and a ratio of approximately 3:1 (mutant: wild type) was observed. To confirm whether a single copy of the autonomous I element (AIE) was responsible for the activation of nearby genes, Southern blot analysis was performed. Genomic DNA was extracted from F1 plants, the parental line and wild type plants digested with *Ecor*RI and probed with the *BAR* gene. For all plants that showed the *twt1-D* phenotype only a single hybridising band was observed. Plants that did not show the presence of this AIE band had a wild type phenotype. Based on this co-segregation and the inheritance ratio, it was concluded that the *twt1-D* mutant is the result of a dominant gain-of-function mutation caused by the presence of a single AIE element (data not shown).

Figure 1. Phenotypes of the *twt1-D* mutant plants compared to wild type at various stages of plant development. **(A, C, E, G, I, J)** wild type and **(B, D, F, H, I, J)** *twt1-D* plants. **(A)** Wild type Col-0 siliques showing two straight fused carpels whereas the *twt1-D* siliques **(B)** show a helical growth. **(C)** Wild type rosette leaves having the petiole and the blade positioned horizontally whereas in the *twt1-D* plants **(D)**, they are twisted in a counterclockwise direction in an angle of 45 degrees. **(E)** Inflorescence of a wild type plant. **(F)** The *twt1-D* inflorescence also turns in the same direction as other organs. **(G)** Adult wild type plant **(H)** Adult *twt1-D* plant showing a loss of apical dominance. **(I)**

Seedling growing on a agar plate. Starting from the left side of the plate, the first 4 plants are *twt1-D* and at the right, 3 wild type seedlings grown at the same time. **(J)** Same plate as **(I)** but at later stage. It can be noted that *twt1-D* roots are bushier when compared to wild type due to more pronounced secondary branching. ***Colour picture, see Appendix.***

TWT1 encodes a transmembrane protein

Because the *twt1-D* mutant was obtained by transposon-based activation tagging using a 4x*35S* enhancer construct as activator, the misexpressed flanking genes could be identified by TAIL-PCR (Liu et al., 1995; Liu and Whittier, 1995; Tsugeki et al., 1996). Sequence comparison of the obtained flanking regions and the *Arabidopsis* genome database revealed that the single AIE was located at chromosome 2, between two annotated genes. Gene At2g32270 was positioned around 8.2 kb upstream and gene At2g32280 was 3.6 kb downstream of the 4X*35S* enhancer sequences (Figure 2).

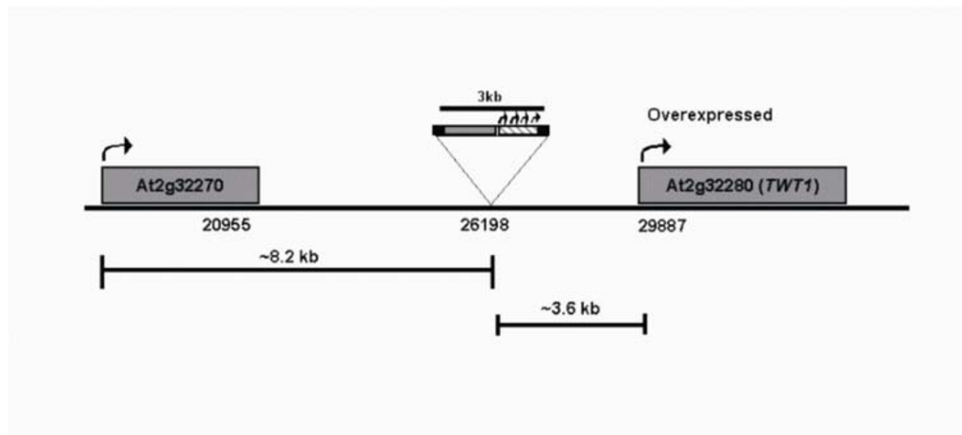

Figure 2. Schematic representation of the *twt1-D* genomic region where the activation insertion element (AIE) was inserted. The insertion was located between two genes at chromosome 2. The distances of the two genes to the 4x*35S* enhancer elements in the AIE are represented by black lines. Gray boxes depict the two genes and the arrows represent the direction of the transcription. Numbers beneath the gray boxes show where the ATG start codon is positioned and the number beneath the triangle represent the insertion point in the BAC clone. The *BAR* resistant gene is indicated by a filled gray box. The small black boxes are the left and the right junction of the AIE element.

No putative open reading frames (ORF) were found between these 2 genes. RNA expression levels of the two candidate genes were checked by Northern hybridisation analysis, using wild type WS-3 and *twt1-D* mutant material from roots (R), rosette leaves (RL), cauline leaves (CL), young flower buds (the top of the inflorescence) (CF), open flowers (OF), stems (ST) and siliques (S). Compared to wild type samples, increased levels of expression of the gene At2g32280 was detected in all tested tissues of the *twt1-D* plant, except in roots (Figure 3A). No difference in expression was observed for the other gene At2g32270. This indicated that the *twisted1-D* phenotype is caused by the overexpression of At2g32280, renamed *TWISTED1* (*TWT1*) (Figure 3). *TWT1* encodes a putative membrane protein with four transmembrane regions (Figure 3B). Using the TAIR database, a closely homologous protein, At4g21310 was identified.

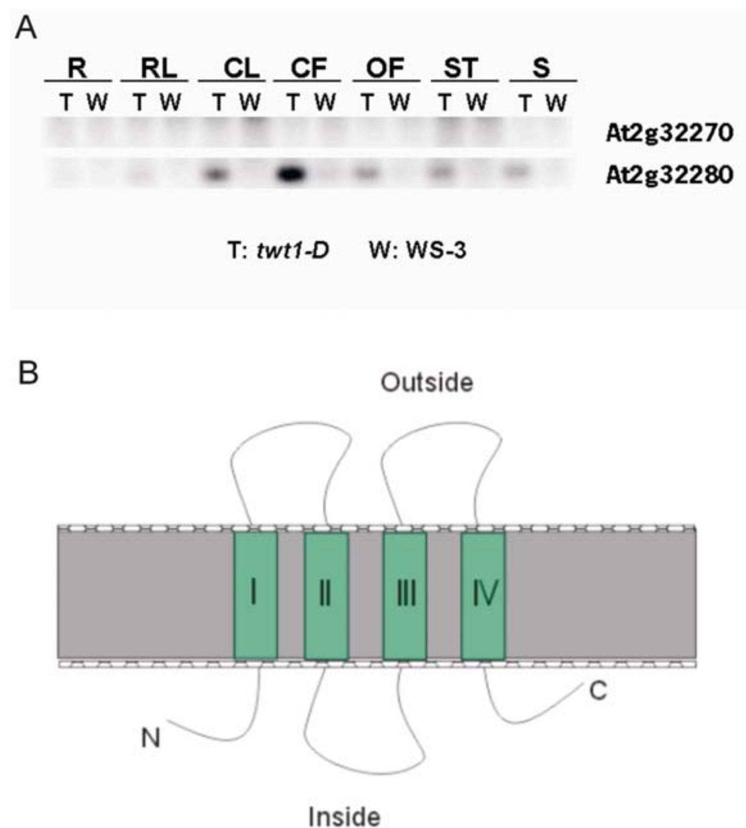

Figure 3. (A) Northen hybridisation blots using material of the *twt1-D* mutant plant (T) and wild type (WS-3 = W). (R) roots, (RL) rosette leaves, (CL) cauline leaves, (CF) top of the inflorescence, (OF) open flowers, (ST) stems and (S) siliques. Top penal was hybridized with the gene At2g32270 and the lower panel, with the gene At2g32280. **(B)** Schematic representation of the TWT1 protein. The four transmembrane domains are represented by filled boxes while the membrane is depicted in gray.

To confirm whether overexpression of *TWT1* is responsible for the morphological changes observed in the gain-of-function *twt1-D* mutant, the predicted *TWT1* cDNA was cloned under the control of the *CaMV35S* promoter. The construct *35S::TWT1* (pARC083) was introduced into *Arabidopsis* wild type Col-0. None of the 17 resistant plants transferred to the greenhouse displayed aberrant growth. Northern blots were performed using the T1 primary transformants to examine the expression levels of the transgene. Wild type Col-0 and the *twt1-D* plant were used as controls. The expression of the transgene (*35S::TWT1*) was increased compared to the wild type, whereas it was similar to the expression level observed in the *twt1-D* plant (data not shown). There are a few possible explanations why the expression of the full-length did not result in the expected phenotype. Firstly, the *twt1-D* phenotype might be background sensitive. The activation tagging phenotype was observed in WS-3 whereas the confirmation was performed in Col-0. Secondly, regions outside of the ORF such as introns or promoter regions could be important for regulation or specificity as observed for *AGAMOUS*-like genes (Sieburth and Meyerowitz, 1997). Thirdly, it is possible that other down or upstream genes are overexpressed along with *TWT1* and therefore, responsible for the phenotype. With these 3 possibilities in mind, two new constructs were made. The first construct, pARC170, includes the complete genomic region from the 4x*35S* enhancers to around 500 bp downstream the last exon of the *TWT1* gene. This construct was 6.5 kb in length and was obtained from the *twt1-D* mutant (Figure 4A). The second construct, pARC173, includes the predicted coding region plus the 3' UTR of *TWT1*. The genomic fragment spans about 1.5 kb from the predicted ATG start codon to 500 bp downstream of the predicted stop codon (Figure 4A). Both constructs were transferred to both Col-0 and WS-3 genetic backgrounds. With regards to construct pARC170, the *twt1-D*-like phenotype was observed for leaves of small plantlets (WS-3 and Col-0) that were still growing on agar plates (Figure 4B). The twisted phenotypes observed in the T1 plants were classified into 2 classes based on the similarity to the *twt1-D* mutant. Mild (M) plants only show the phenotype similarity in rosette leaves and in loss of apical dominance. In severe (S) plants, the twisted phenotype was present in all organs, reminiscent with the *twt1-D* mutant (Figure 4C, D, E). The results are summarised in table 1. The *twisted1-D* phenotype that was observed for the longest construct demonstrates that the overexpression of the *TWT1* gene causes the mutant phenotype of the *twt1-D* gain-of-function mutants.

Only twisting phenotypes belonging to the mild class were observed in plants transformed with construct pARC173 (Figure 4F,G). Because pARC173 begins with the predicted ATG, it was thought that the gene might not be correctly annotated. Therefore, databases were consulted to understand the gene structure of *TWT1*. Most gene prediction software predicted the same ATG start codon of the hypothetical TWT1 protein.

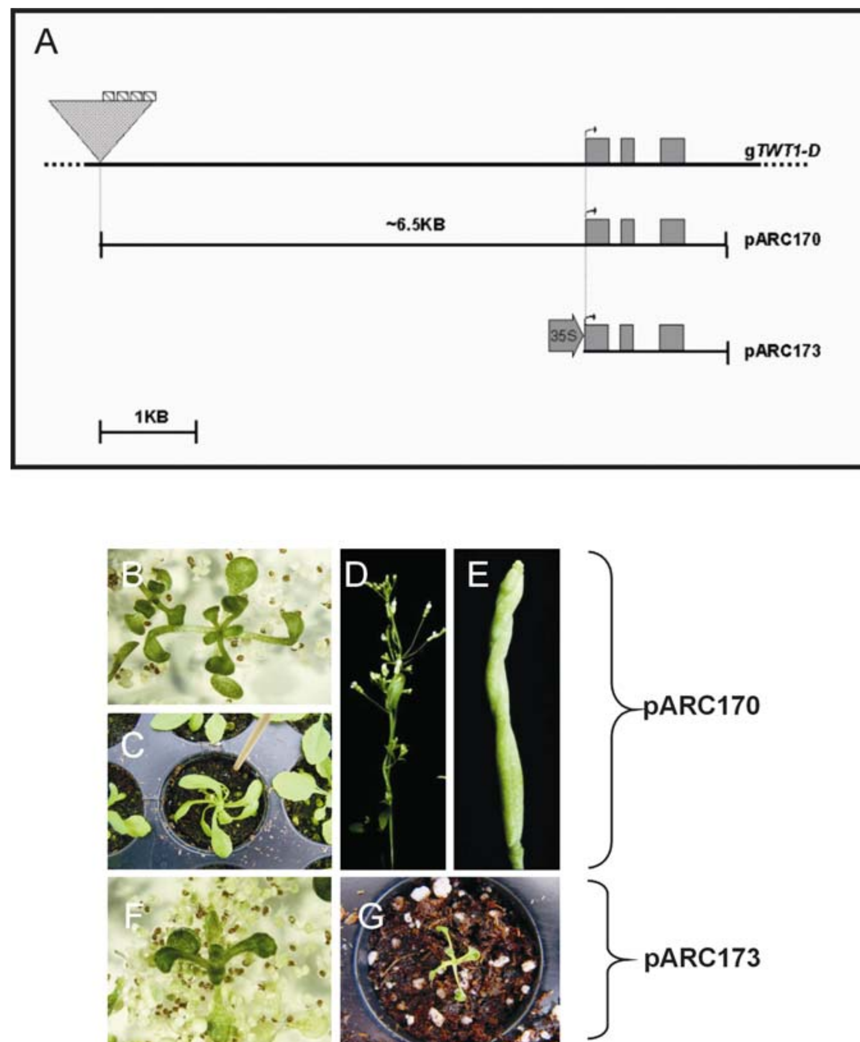

FIGURE 4. **(A)** Schematic representation of the two constructs, pARC170 and pARC173, used in the overexpression studies with *TWT1*. The upper line represents the genomic region of the *twt1-D* mutant with the activation insertion element (dotted triangle) and the *TWT1* gene. Thick black lines are the approximately the genomic region, where the numbers indicate the fragment size. Gray filled boxes are the exons. The arrow on top of the first exon shows the direction of the transcription. Construct pARC170 has the quadruple *35S* enhancer sequence at the 5' end, while the *CaMV35S* (35S) promoter (gray triagle) is upstream the *TWT1* sequence in the construct pARC173. (B - E) pARC170 and (F, G) pARC173 phenotypes. **(B)** pARC170 small seedlings on plate showing the counterclockwise direction of growth of the first leaves. **(C)** pARC170 plant with twisted rosette leaves. **(D)** Inflorescence of a pARC170 plant, depicting the loss of apical dominance and twisted growth. **(E)** Twisted silique of a pARC170 plant. **(F)** pARC173 seedling showing a weak turning *in vitro*. **(G)** Young pARC173 plantlet. ***Colour picture (B-G), see Appendix.***

Table 1: Phenotypes of plants that were transformed with construct pARC083 in Col-0, pARC170 in WS-3 and in Col-0, pARC173 in WS-3 and in Col-0. Number of plants that showed the mild (M) or severe (S) twisted phenotype is indicated.

Construct	Number of plants transferred to the greenhouse	Number of plants with the M, or S phenotype
pARC083 in Col-0	17	0 (M); 0 (S)
pARC170 in WS-3	80	10 (M); 50 (S)
pARC170 in Col-0	40	18 (M); 18 (S)
pARC173 in WS-3	29	16 (M); 0 (S)
pARC173 in Col-0	27	9 (M); 0 (S)

However one prediction, GlimmerA, predicted two extra exons upstream and a shorter last exon (Figure 5). Thus, to get more reliable data, a rapid amplification of cDNA ends (RACE) was performed, to amplify the 5' of the *TWT1* gene. Interestingly, the results obtained for both WS-3 and Col-0 were the same as the EST annotated in the database and the cDNA used in this study (results not shown). These results suggest that there are motifs present in the promoter region that are responsible for the severe *twisted1-D* phenotype.

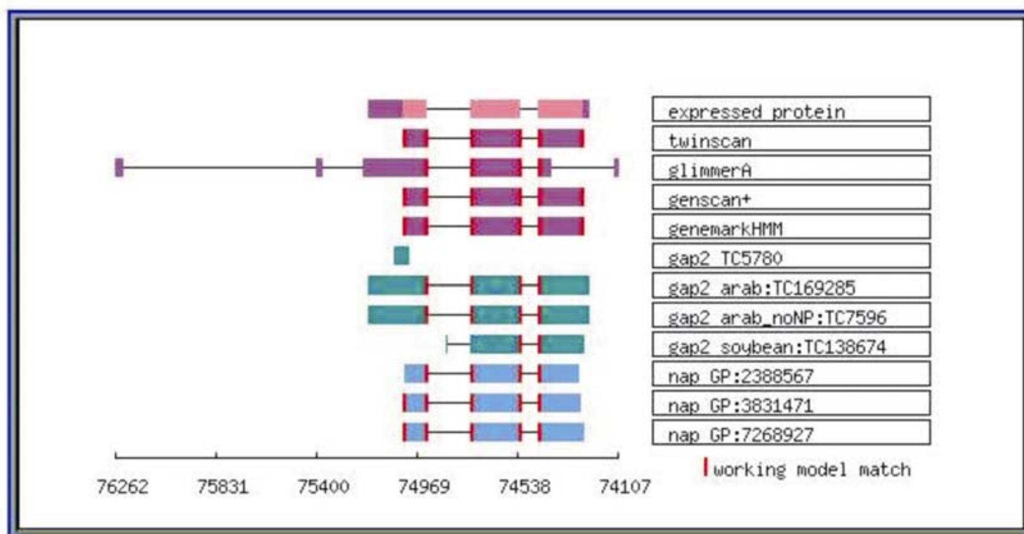

Figure 5. Gene structure predictions of the *TWT1* gene using different software packages. The figure was generated using the TIGR database (www.tigr.org/tdb/e2k1/ath1/LocusNameSearch.shtml).

***TWT1* is expressed in the vascular tissues**

Based on the Northern hybridisation results (Figure 3A) the *TWT1* gene is expressed at very low levels. Both *in situ* hybridisations and promoter-reporter experiments were performed to determine the expression pattern of *TWT1*.

About 2.0 kb of the putative *TWT1* promoter region was fused to the β-glucuronidase (*GUS*) gene and transgenic plants were analysed for GUS expression. GUS activity was observed throughout the entire plant (Figure 6A). A detailed analysis revealed that the activity is concentrated in the vascular tissues, xylem and phloem and very strong in trichomes present on the surfaces of rosette and cauline leaves (Figure 6B, C). In flowers, *pTWT1::GUS* expression is observed in vascular tissues of all organs and is particularly apparent in the stamen filaments and the pedicel (Figure 6D). In the silique, high levels of GUS activity is seen in the vascular tissue present in the septum and the funiculus (Figure 6E, F).

In situ RNA hybridisations were also performed to localise the *TWT1* RNA expression at the cellular level. Consistent with the *pTWT1::GUS* results, the *TWT1* expression pattern in the flowers is strongest in the vascular cells of the pistil (Figure 6G,H). *TWT1* expression is also observed at earlier stages primarily in the petal, stamen and carpel primordia in which no vascular tissues have been differentiated yet (Figure 6G). At later stages (Figure 6H), patches of vascular tissues visible in the longitudinal section, shown in Figure 6H, exhibit hybridising signals.

***twt1* knockout mutant shows no phenotype**

twt1-D is a dominant gain-of-function mutant, which shows substantial changes in plant architecture. To get further insights in the function of *TWT1*, loss-of-function mutants were obtained from the SALK and the former GARLIC (SAILS) populations. From each population, two T-DNA insertion lines were isolated and screened for aberrant phenotypes. Homozygous plants were identified and the presence of the insertions in *TWT1* was confirmed by sequencing (results not shown). No alterations were observed in any of the *twt1* mutants grown under normal greenhouse conditions. Although *TWT*1 expression was not tested, it is expected that the mutant alleles analysed represent real null alleles. The insertions in the lines were either in the first intron (line N523737 and GARLIC_893_G06.b.1b.Lb3Fa), or in the second exon (line GARLIC_237_C09.b.1a.Lb3Fa) or in the third exon (line N547972). The absence of an obvious phenotype might be the result of the presence of redundant genes. Therefore, loss-of-function mutants of 2 alleles of the closest *TWT1* homolog, AT4g21310, were selected from the SALK database. Phenotypic analysis of these mutant lines revealed no aberrant phenotype (data not shown). Double mutants may be necessary to overcome the redundancy phenomena and to observe a mutant phenotype.

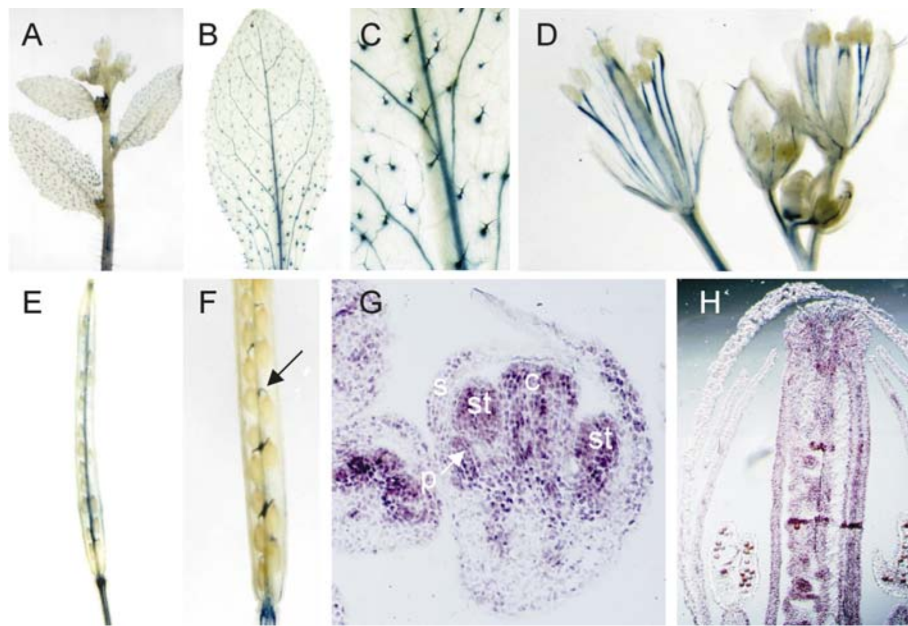

Figure 6. GUS expression driven by the *TWT1* promoter and *in situ* mRNA accumulation analysis. (A-F) GUS activity at various developmental stages of *pTWT1::GUS* lines. **(A)** Inflorescence with cauline leaves. GUS staining is observed in trichomes and secondary buds. **(B)** Detail of a cauline leaf showing expression in vascular tissues and trichomes. **(C)** Detail of (B). **(D)** Flowers of *pTWT1::GUS* plant. **(E)** Silique with high GUS activity in septum, receptacle and funiculi. **(F)** Part of a silique illustrating expression in funiculi (arrow). **(G and H)** *In situ* hybridisation using an antisense DIG-labeled *TWT1* probe on longitudinal sections. **(G)** Young wild type flower buds with floral organ primordia. s=sepal; p=petal; st= stamen; c=carpel. **(H)** Part of a flower at later developmental stage just before anthesis. High expression levels are present in the pistil and patches of vascular tissues in petals. ***Colour picture, see Appendix.***

TRN1* is epistatic to *TWT1

It has been reported that a loss-of-function mutant, *tornado1-1* (*trn1-1)* shows besides other effects, twisted organs (Cnops et al., 2000). To determine whether *TWT1* and *TRN1* might be involved in the same genetic pathway *trn1-1 twt1-D* double mutants were generated. Because *trn1-1* is very dwarfish and difficult to manipulate, *trn1-1* heterozygous plants were used in a cross with a homozygous *twt1-D* plant. F2 segregating plants were analysed for any additive affect. Additional phenotypes in *trn1-1/twt1-D* double mutant plants compared to *trn1-1* single mutants were not observed. PCR was used to determine which plants with a *trn1-1* phenotype were double mutants (results not

shown). The double mutants displayed an identical phenotype as *trn1-1* single mutants (Figure 7B), suggesting that *trn1-1* is epistatic to *twt1-D*.

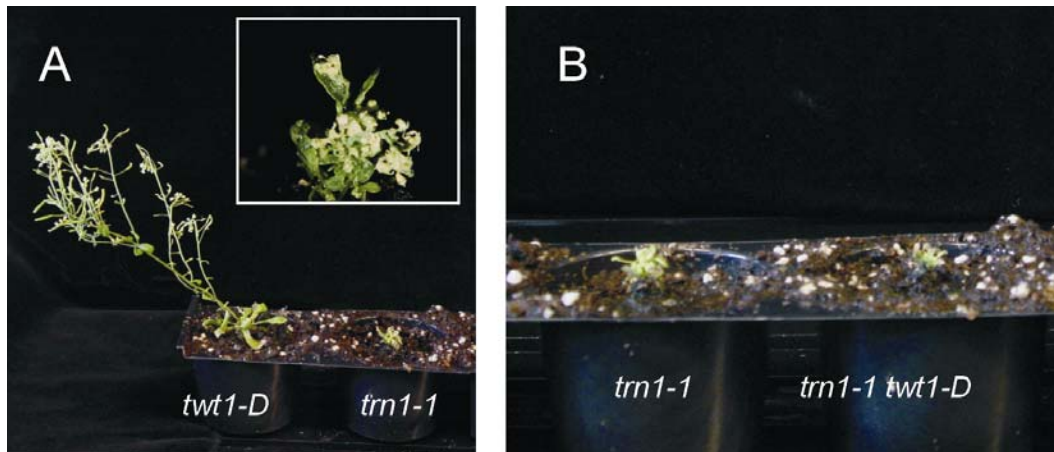

Figure 7. (A) *twt1-D* and *trn1-1* mutant plants. The highlighted square at the top right side is an enlargement of the *trn1-1* mutant. **(B)** *trn1-1 twt1-D* double mutant plant compared to the *trn1-1* single mutant in the same condition of growth. Double mutant plant is identical to the single *trn1-1*, indicating that *trn1-1* is epistatic to *twt1-D*. ***Colour picture, see Appendix.***

Discussion

twt1-D* mutation is due to the overexpression of the *TWT1

In an activation tagging screening, the *twt1-D* mutant was isolated with an aberrant phenotype. To confirm that the mutant phenotype is due to the upregulation of the endogenous *TWT1* gene, recapitulation of the *twt1-D* phenotype was performed. At first attempt, the predicted *TWT1* cDNA was fused to the *35S* promoter and although the overexpression of the transgene was confirmed, no twisted-like phenotype was observed. In a second attempt, two new constructs were used. One containing a 6.5 kb genomic fragment containing the predicted *TWT1* gene and the 4x*35S* enhancers obtained from the original *twt1-D* mutant and another beginning from the ATG start codon to the same position downstream the gene (pARC173). Using these two new constructs, several twisted-like phenotypes were obtained, such as twisted rosette leaves and loss of apical dominance. The severe twisted phenotype, which includes twisted siliques and stems, was only observed with construct pARC170 that contained the 6.5 kb genomic fragment. Construct pARC173, with the *35S* promoter instead of the endogenous promoter, yielded only weak phenotypes, suggesting that the enhancement of the endogenous promoter is responsible for the severe twisted phenotype. It is known that the *CaMV35S* promoter,

which is generally used as a constitutive promoter, still exhibits spatial and temporal specificity. Although activation tagging could lead to either overexpression or ectopic expression of the nearby gene, the results reported in this thesis and by Marsch-Martinez et al. (2002) suggest that overexpression is more common. In the cases of the *twt1-D* mutant and the lines transformed with pARC170, the *TWT1* gene, which is active in vascular tissues, appears to be overexpressed rather than ectopically expressed in other tissues. This could explain the differences observed between the phenotypes obtained with the endogenous promoter on the one hand, and the constructs containing the *35S* promoter on the other hand.

TWT1 and auxin transport

A loss-of-function mutant, involved in auxin transport and vascular patterning, *lopped* (*lop1*) (Carland and McHale, 1996), has been described in having a twisted silique phenotype, including twisting of leaf petioles and pedicels of the flowers. In this mutant the epidermal transport is disrupted, which leads to accumulation of indoleacetic acid (IAA). Subsequently, aberrant cell expansion occurs, which is strongly correlated with the twisted roots in *lop1*. This is also the reason of the twisted phenotype seen in *lop1* aerial organs (Carland and McHale, 1996). Another loss-of-function mutant, *trn1-1* (Cnops et al., 2000) has also an abnormal growth pattern, being dwarf and the 'twisting' effect is even more drastic than in either *lop1* or *twt1-D*. These effects have been attributed to a defect in the formation of an auxin maximum, strongly suggesting that polar auxin transport is also involved in the *trn1-1* mutant phenotype. Whether a similar auxin transport effect is the case in the *twt1-D* plants is not known. Nevertheless, the expression of the gene in the vascular tissue and the molecular nature of the protein being a putative membrane spanning protein are in line with a putative role in hormonal transport. Furthermore, the 'twisted' nature of the organs in *twt1-D* points to a disturbed pattern of cell elongation, which can be caused by changed auxin levels. Obviously, more experimental data are required to be more elusive about the molecular action of *TWT1*.

Role of *TWT1* in vascular tissues

TWT1 is expressed in all plant organs, from rosette leaves to flowers. Based on the *pTWT1::GUS* expression data it was concluded that *TWT1* expression is localised in the vascular system, although it is not known in which cells specifically. Recently, it has been reported that *APL*, a gene encoding a MYB transcription factor regulates vascular tissue identity (Bonke et al., 2003). The *APL* expression pattern is consistent with its role in phloem development. Ectopic expression of *APL* in the vascular bundle inhibits xylem development. Interestingly, it has been reported that this loss-of-function mutation causes lethality in seedlings but does not display any twisting of organs. Because the *twt1*

knockout mutant does not show an aberrant phenotype, the *APL* and *TWT1* genes are difficult to compare, although the are likely expressed in the same tissues. Based on the assumption that *TWT1* encodes a membrane protein it is more likely that *TWT1* plays a role in transport rather than having a homeotic function, which is assigned to *APL*.

pTWT1::GUS expression is not restricted to the vascular system exclusively. High GUS activity was also observed in trichomes, which are hair-like structures that consist of a single cell. This cell undergoes a series of cell biological events, including endoreduplication, cell expansion and outgrowth (Hülskamp et al., 1994). Furthermore, the *in situ* hybridisation data revealed expression in floral organ primordia and various tissues of the pistil. Interestingly, *twt1-D* shows a more dramatic phenotype in pistils and siliques with an extraordinary twisting, an observation that fits with the idea that *TWT1* is overexpressed in these tissues of the *twt1-d* mutant.

There is a striking correlation between auxin action and the expression pattern of *TWT1*, which points again to a link between the TWT1 membrane protein and the auxin pathway. It is known that auxin plays a major role in phloem and xylem differentiation and cell expansion, which are processes that occur in the vascular tissues, trichomes and primordial cells.

Interestingly, the twisting phenotype in the *twt1-D* mutant has always a right-handed helical direction in all organs. In contrast, overexpression of *WAVE-DAMPENED2* (*WVD2*) and *WVD2-LIKE1* (*WDL1*) resulted in right-handed turns in roots and etiolated hypocotyls, whereas petioles were twisted left-handed (Yuen et al., 2003). Mutations also in *SPIRAL2 (SPR2)*, *LEFTY1* and *LEFTY2* caused twisting in all organs in the same direction (Furutani et al., 2000; Thitamadee et al., 2002). It has been reported that in *lefty1* and *lefty2* mutants the cortical microtubule stability is reduced, which results in a left-handed organ twisting (Thitamadee et al., 2002). Whether the microtubular organisation is impaired in the *twt1-D* mutant and how it relates with the normal function of the TWT1 protein in vascular tissue development or functioning remains to be determined.

Gain-of-function mutants may shed light on the function of a particular gene, although one should be careful in drawing conclusions because the gene is misexpressed. Unfortunately, no obvious mutant phenotypes were observed for the *twt1* loss-of-function lines, which would be very informative for its function. Either a very subtle phenotype is apparent, but has not been noticed, or *TWT1* is highly redundant with the related gene At4g21310. Also a T-DNA insertion in this gene did not affect the phenotype of the plant. Thus, a double mutant combination *twt1 at4g21310* has to be tested to get more insight in their functions.

In conclusion, here we report about a novel gene, which encodes a putative membrane spanning protein. Its expression pattern and gain-of-function phenotype suggest a role in vascular tissue development. Furthermore, one could speculate a link between

auxin transport or perception and *TWT1*. The 'twisted' nature of the organs in the overexpression mutant might be caused by impaired cellular organisation and abnormal cell elongation. More detailed analyses are necessary to unravel these cellular defects and the relation with auxin.

Acknowledgments

The authors thank Liam Dolan for providing *trn1-1* seeds, Andy Pereira's group for discussion and for generating the activation tagging population. Thanks to all members of Gerco Angenent's lab for ideas, suggestions and contributions during the entire period of this work, Faye Rosin for comments on the manuscript and Maarten Koornneef and Mark Aarts for fruitful discussions. This work has been financially supported by CAPES-Brazil (BEX 1519/98-0)

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CHAPTER 8

This thesis describes the study the molecular control of several aspects of plant development in *Arabidposis thaliana*. *Arabidopsis* was chosen as model species for this study because it has several advantages, such as the small genome size, short generation time, abundant number of seeds, and easy transformation procedure. The selected strategy was to isolate mutants that have an altered morphology, in particular focused on flower and silique mutants. Obviously, the best way would be to use gene disruption, which subsequently affects gene function in the tissues were it is active. Gene knockout is considered to be the major component of the functional genomics toolbox. Large mutant *Arabidopsis* populations have been produced by T-DNA insertions (Alonso et al., 2003). However, many insertion mutants do not provide an informative mutant phenotype (Bouche and Bouchez, 2001). This lack of mutant phenotype under "normal" conditions is mainly attributed to gene redundancy, caused by the extensive genome duplications in *Arabidopsis* (*Arabidopsis* Genome Initiative, 2000). Therefore, new strategies to overcome this drawback in generating mutants have been developed. One of them is the generation of gain-of-function mutations by activation tagging strategies. In this work, a phenotypic screening was performed on an activation tagging population, leading to the isolation of three interesting mutants affected in plant development. The molecular basis for the gain-of-function mutations was determined and shed light on the molecular regulation of developmental processes. Surprisingly, these gain-of-function mutants resembled know loss-of-function mutants, which pointed to a general phenomenon in the control of biological processes: antagonistically acting genes control major switches in plant development, such as flower induction and differentiation of cells. Therefore, despite being overexpressing mutants, they give clues about the normal function of the genes affected.

Activation tagging - the isolation of plant architecture mutants

One of the best characterized and often used activation tagging population is the T-DNA activation tagging population generated by Weigel and co-workers (2000). In this system, a large collection of *Arabidopsis* plants, transformed with a binary vector containing the quadruple cauliflower mosaic virus (*CaMV*) *35S* enhancer, was created. This T-DNA based system has since then been used by many research groups for obtaining interesting mutants in several pathways (Borevitz et al., 2000; Ito and Meyerowitz, 2000; Lee et al., 2000; van der Graaff et al., 2000; Weigel et al., 2000; Huang et al., 2001) and also as a tool to characterize gene families (Nakazawa et al., 2003). Although very successful, the observed frequency of dominant morphological mutants was only 0.1%, making this method 10-fold less effective than transposon-based activation tagging, a system which is reported to generate about 1% of aberrant plants (Marsch-Martinez et al.,

2002). Therefore, we aimed to elucidate the reason for this disadvantage (Chapter 4). With the study described in chapter 4, it was possible to identify methylation of the 4x*35S* enhancer sequences, which was correlated with a transcriptional silencing. This could be a likely cause for the low frequency of visible mutant phenotypes observed with the T-DNA based activation tagging approach. Because the frequency was at least 10 times higher with a transposon-based system, which most likely does not induce silencing of the enhancers, we chose to screen such a population. This population was generated by Pereira's research group at Plant Research International. From this screening 3 gain-of-function mutants were obtained which are described in chapters 5, 6 and 7 of this thesis. In these chapters, their molecular and genetics features are presented and discussed.

One of the biggest advantages of using activation tagging is that it can overcome the functional redundancy present in the *Arabidopsis* genome. Indeed, for all 3 genes defined by activation-mutants described in this thesis, loss-of-function mutations lack any phenotype. In Chapter 5 the *downwards siliques1-D* (*ds1-D*) mutant is described. This gain-of-function mutant shows a conspicuous phenotype. The mutation has an affect on inflorescence architecture. Flowers and siliques are bending downward and the pedicels are reduced in length. Although this may give some clues on the function of *DS1*, a knockout mutant would be very informative. Only in a double mutant with another LOB domain gene *AS2,* morphological abnormalities were observed. In double mutant plants, blade cells of the petals become more petiole-like, thereby altering the proximal-distal symmetry of the petal.

A similar strategy was also used for the other dominant gain-of-function mutant, *needle1-D* (*ndl1-D*) (chapter 6). The normal formation of valve tissues was altered in this gain-of-function mutant. When the corresponding *ndl1* loss-of-function mutant was analysed, no phenotype was seen. The generation of double mutants with a few candidate paralogues is in progress. A similar situation was also found for the third mutant (chapter 7). In the *twisted1-D* (*twt1-D*) mutant all organs are twisted with the most severe phenotype observed in the siliques. In this case, four knockout alleles of *twt1* were obtained and none of them showed a mutant phenotype, reinforcing the idea that at least double mutants are required to obtain a mutant phenotype.

We have shown that activation tagging is a powerful approach to identify new mutants. However, the use of *35S* enhancers as activator elements may generate unwanted side effects, such as lethality or many pleiotropic defects in the entire plant. In chapter 3, we investigated the possibility to create tissue specific activation. Tissue specific activation tagging is an alternative for the generally applied activation tagging approach, which uses the 4x*35S* enhancers. For the creation of specific mutations in a desired tissue or organs, the 4x*35S* enhancer is not very attractive, because it may induce aberrations in more plant tissues (Weigel et al., 2000; Marsch-Martinez et al., 2002). To test this hypothesis we have

characterized a tissue specific enhancer that might be applicable for the generation of specific mutants. As example, we have used an enhancer fragment of the *SHATTERPROOF2* (*SHP2*) gene, which is expressed predominantly in the dehiscence zone. The study reported in chapter 3 revealed that the enhancer acts specifically in the dehiscence zone, when combined with the tester promoter *FBP*1 or the minimal *35S* promoter. This activity was not influenced by its orientation relative to the promoter and it was still functional at 2.0 kb, the longest tested distance. Based on these results, the *SHP*2 enhancer fragment might be used in an efficient tissue-specific activation tagging approach to identify new *Arabidopsis* mutants with an altered dehiscence zone formation.

Conclusions and perspectives

The aim of this thesis was to isolate novel mutants that might help in understanding plant architecture, especially flower and silique development. The use of the transposon activation tagging allowed us to obtain three interesting gain-of-function mutants. In combination with expression analysis and information about other loss-of-function mutants with remarkably similar phenotypes, we gain valuable information about the role in plant development of three novel genes. These overexpression mutants (*ds1-D*, *ndl1-D* and *twt1-D*), appear to be more informative than the single knockout mutants, demonstrating the power of activation tagging strategies.

The information gained by the research reported in this thesis gives a fragmentary view on a few important regulatory networks in plant development. However, with the enormous *Arabidopsis* resources and X-omics toolbox available and the worldwide input in *Arabidopsis* research it will be soon possible to link the networks together. With the components of the networks identified and the molecular interactions elucidated, a new challenge in biology, *systems biology*, can start.

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SUMMARY

Studying plant architecture is not only important for systematic and taxonomic classification, but also for agronomic reasons. It influences e.g., cultivation characteristics leading to higher yield and quality of the product. Thus, a better understanding of plant architecture may lead to ways to control and modify agronomically important traits.

Arabidopsis thaliana is a weed that has several advantages for plant science including its small genome size, short generation time, abundant number of seeds, easy transformation system, and, recently a completed genome sequence. These advantages have made this plant the model species of choice in many areas of plant science.

Activation tagging is a powerful tool to identify new mutants. It has emerged as an alternative approach for gene function analysis, generating gain-of-function mutations. It is based on the use of the quadruple cauliflower mosaic virus (*CaMV) 35S* enhancer sequences as activator elements.

This thesis reports the study on the molecular control of plant architecture, using mutants obtained by an activation tagging-based approach in *Arabidopsis thaliana*.

Chapter 1 of this thesis briefly describes the concepts of plant architecture and the tools to obtain *Arabidopsis* mutants affected in development. It summarizes the history of *Arabidopsis* research and introduces the techniques utilized in these studies. It concludes with the aim of these studies and an outline of the chapters.

Chapter 2 is a literature review about important molecular switches involved in major steps of plant development. Several examples of antagonistically acting genes are discussed, which gives an impression how processes are controlled at the molecular and genetic level.

Chapter 3 reports an example of tissue specific activation, which aims to generate tissue or organ-specific mutations. Tissue specific activation tagging may overcome the side effects caused by the use of the *CaMV35S* enhancers as general activator elements. As example for this study, enhancer sequences of the S*HATTERPROOF2* (*SHP2*) gene was used. The *SHP2* gene is involved in the formation of the dehiscence zone, and it is expressed in the pistil and during silique development. Its putative enhancer sequences were fused to the test promoter of *FLORAL BINDINDG PROTEIN1* (*FBP1*) or the *35S* minimal promoter. It was shown that this *SHP2* enhancer in combination with either the *FBP1* promoter or minimal *35S* promoter drives dehiscence zone specific expression, independent of the distance or orientation towards the promoters. These results indicate that this enhancer may be useful for a dehiscence zone specific activation tagging approach to create new *Arabidopsis* pod shatter mutants.

Activation tagging does not have only advantages. One of the disadvantages is reported in **chapter 4**. In this chapter, a possible cause for the low efficiency of the T-DNA

based activation tagging approach is investigated. Large populations of individual *Arabidopsis* plants carrying a quadruple *35S* enhancer are frequently used for mutant screenings. However, the frequency of occurrence of mutants with altered morphology is low. Our results indicated that the low success rate of this approach is correlated with the methylation of the *35S* enhancer sequences. All plants containing more than a single T-DNA insertion showed methylation of the *35S* enhancer and a dramatic decrease in *35S* enhancer activity. Given the low success rate of T-DNA based activation tagging, an alternative approach was followed. The frequency of developmental mutants using a transposon based activation tagging was 10-fold higher than the T-DNA based system. Therefore, phenotypic screenings were performed with a transposon-tagged population and three interesting mutants with altered morphology were obtained.

Chapter 5 deals with the *downwards siliques1* (*ds1-D*) mutant. In the *ds1-D* mutant, internodes are shorter and the lateral organs such as flowers are bending downwards, similar to the known loss-of-function mutant, *brevipedicellus* (*bp*). Expression studies showed that the *ds1-D* mutant was a gain-of-function mutant caused by the overexpression of the *DS1* gene. Analysis of the loss-of-function mutant *ds1* revealed no mutant phenotype, suggesting gene redundancy. Therefore, double mutants with *DS1* and its closest homolog, *AS2* were generated. With the experiments described in this chapter, it was possible to conclude that these two members of the LATERAL ORGAN BOUNDARY (LOB) gene family act redundantly to control cell fate determination in the *Arabidopsis* petals.

The analysis of another gain-of-function mutant, *needle1-D* (*ndl1-D*) affected in the *NEEDLE1* gene, which encodes a basic Helix-Loop-Helix (HLH) transcription factor is described in **chapter 6**. Overexpression of *NDL1* driven by the 4x*35S* enhancers present in the transposable element altered the normal formation of valve tissues in *Arabidopsis* siliques. This resulted in the formation of a pin-like structure replacing the two fused carpels of the wild type pistil. Interestingly, the phenotype of the *ndl1-D* mutant resembled the phenotypes of loss-of-function mutants which are affected in the regulation of carpel development and auxin tranport, such as *ettin*, *pinoid* and *pin-formed*. The loss-of-function mutation in *NDL1* did not show any phenotype, indicating that other members of the HLH family of transcription factors are acting redundantly with *NDL1*. *NDL1* expression is restricted to pistil/silique tissues. Moreover, auxin levels in the *ndl1-D* mutant were elevated, mainly in the altered gynoecium, suggesting that the gene plays a role in auxin transport during carpel development in *Arabidopsis*.

Chapter 7 describes the results of the study on the *TWISTED1* (*TWT1*) gene, identified by the third gain-of-function mutant, *twisted1-D*. In this mutant, all organs become twisted with the most severe phenotype observed in the siliques. Expression analysis revealed that *TWT1* is expressed in the vascular system of the entire plant. Similar

to *DS1* and *NDL1*, the loss-of-function mutation in *TWT1* did not show any phenotype, suggesting functional redundancy. *TWT1* encodes a putative transmembrane protein of unknown function. The *twt1-D* phenotype combined with the expression analysis of *TWT1* suggest that this gene is involved in vascular tissue development.

Chapter 8, is a general discussion of the work described in this thesis. The results of all chapters are connected and a general assessment of their contribution to our understanding of plant development is presented.

SAMENVATTING

Het bestuderen van plant architectuur is niet alleen belangrijk voor systematische en taxonomische classificatie, maar is ook belangrijk voor agronomische redenen. Het beïnvloedt bijvoorbeeld de teelt van gewassen waardoor de opbrengst verhoogd en kwaliteit van het product verbeterd kan worden. Het beter begrijpen van de architectuur van een plant kan dus leiden tot het verbeteren van agronomisch belangrijke eigenschappen.

Arabidopsis thaliana is een onkruidje dat meerdere voordelen heeft voor het bestuderen van planten, waaronder een klein genoom, korte generatietijd, grote aantallen zaden, efficiënt transformatiesysteem en recentelijk een volledig gesequenced genoom. Deze voordelen hebben deze plant tot het algemene modelsysteem gemaakt voor plantenonderzoek.

Activatie tagging is een krachtige methode voor het identificeren van nieuwe mutanten. Het heeft zich ontwikkeld tot een alternatieve methode voor genfunctie analyse, waarbij *gain-of-function* mutanten gecreëerd kunnen worden. Het is gebaseerd op het gebruik van een activatie element bestaande uit viermaal de cauliflower mosaic virus *(CaMV) 35S* enhancer sequentie. Dit proefschrift beschrijft de studie van de moleculaire regulatie van de plant architectuur door gebruik te maken van een activatie-tagging strategie in *Arabidopsis thaliana.*

Hoofdstuk 1 beschrijft kort de verschillende concepten die ten grondslag liggen aan plant architectuur en de middelen om *Arabidopsis* ontwikkelingsmutanten te verkrijgen. Het vat de geschiedenis van *Arabidopsis* samen en introduceert de technieken die gebruikt zijn in deze studie. Het eindigt met de doelstelling van dit onderzoek en een overzicht van de hoofdstukken.

Hoofdstuk 2 geeft een literatuur overzicht over belangrijke moleculaire schakelaars die betrokken zijn bij de belangrijkste stappen van de plantontwikkeling. Verschillende voorbeelden van genen die antagonistisch werken, worden besproken. Dit geeft een indruk over hoe processen gecontroleerd worden op moleculair en genetisch niveau.

Hoofdstuk 3 beschrijft de ontwikkeling van een weefselspecifieke activatie tagging aanpak met als doel om weefsel- of orgaanspecifieke mutaties te genereren. Weefselspecifieke activatie tagging kan de nadelen van het gebruik van de algemene *CaMV 35* enhancer voorkomen. Als voorbeeld voor deze studie zijn enhancer sequenties van het *SHATTERPROOF2* (*SHP2*) gen gebruikt. Het *SHP2* gen is betrokken bij de vorming van de dehiscence zone en komt tot expressie in de stamper en gedurende de ontwikkeling van het houwtje. De veronderstelde *SHP2* enhancer sequentie werd gefuseerd aan de test-promoter van het *FLORAL BINDING PROTEIN 1 (FBP1)* gen of de minimale *35S* promoter. De resultaten laten zien dat de *SHP2* enhancer in combinatie met één van de

promotoren een dehiscence specifieke activiteit vertoont die onafhankelijk is van de afstand of oriëntatie t.o.v. de promoter. Mogelijk kan een dergelijk systeem met een dehiscence zone specifieke enhancer gebruikt worden voor het genereren van mutanten, waarin het openspringen van de houwtjes veranderd is.

Activatie tagging heeft niet alleen voordelen, een van de nadelen wordt besproken in **hoofdstuk 4.** In dit hoofstuk wordt gekeken naar de mogelijk oorzaak van de lage frequentie aan mutanten verkregen via een 4x*35S* enhancer aanpak. Onze resultaten geven aan dat de lage slagingskans van deze aanpak gecorreleerd is aan de methylering van de *35S* enhancer sequenties. Alle planten die meer dan één T-DNA insertie hadden, vertoonden methylering van de *35S* enhancer en een drastische afname van de *35S* enhancer activiteit. Gezien de lage slagingskans van de T-DNA gebaseerde activatie tagging is er een alternatieve route gevolgd. De frequentie van ontwikkelingsmutanten verkregen met een activatie tagging aanpak gebaseerd op transposons bleek namelijk 10 maal hoger te liggen dan de T-DNA aanpak. Daarom werd een transposon tagging populatie gescreend op mutanten en werden drie interessante ontwikkelingsmutanten gevonden.

Hoofdstuk 5 behandelt de *downwards silique1 (ds1-D)* mutant. In de *ds1-D* mutant zijn de internodiën korter en buigen de laterale organen zoals bloemen naar beneden. Deze mutant lijkt op de knockout mutant *brevipedicellus (bp).* Expressie studies lieten zien dat de *ds1-D* mutant een *gain-of-function* mutant is waarin *DS1* tot overexpressie is gebracht. De knockout mutant van *DS1* laat geen afwijkend fenotype zien, waaruit geconcludeerd kon worden dat er waarschijnlijk spraken is van redundantie. Daarom werden dubbelmutanten van *ds1* en de naaste homoloog *as2* gegenereerd. Uit deze experimenten kon geconcludeerd worden dat deze twee leden van de *LATERAL ORGAN BOUNDARY (LOB)* familie redundantie vertonen voor de regulatie van celidentiteit in *Arabidopsis* kroonbladeren.

De analyse van een ander *gain-of-function* mutant, waarin het *NEEDLE1 (NDL1)* gen tot overexpressie is gebracht, wordt beschreven in **hoofdstuk 6**. Overexpressie door de 4x*35S* enhancers aanwezig in het transposon element verandert de normale vorming van de vruchtbladeren van een *Arabidopsis* houwtje. Dit resulteert in een pin-achtige structuur i.p.v de twee gefuseerde vruchtbladeren. Interessant te noemen is dat het fenotype van de *ndl1-D* mutant lijkt op het fenotype van knockout mutanten, die gestoord zijn in de regulatie van vruchtbladontwikkeling en auxine transport, zoals *ettin, pinoid* en *pin-formed*. *De loss-of-fucntion* mutatie in *NDL1* gaf geen fenotype, mogelijk als gevolg van redundantie met andere leden van de HLH transcriptiefactor familie. De expressie van *NDL1* is beperkt tot stamper en houwtjes. Verder was de auxine concentratie in de gemuteerde stamper verhoogd t.o.v. wild type, waaruit gesuggereerd kan worden dat het

gen een rol speelt bij het transport van auxine tijdens de stamperontwikkeling in *Arabidopsis*.

Hoofdstuk 7 beschrijft de resultaten met het *TWISTED1 (TWT1)* gen, dat geïdentificeerd is op basis van de derde *gain-of-function* mutant *twt1-D*. In deze mutant zijn alle organen gedraaid wat vooral te zien is in het houwtje. Expressie analyse gaf aan dat het gen tot expressie komt in de vaatbundels van de plant. Evenals *DS1* en *NDL1* gaf de knockout mutant van *TWT1* geen fenotype. Het *TWT1* gen codeert voor een mogelijk membraam eiwit waarvan de functie niet bekend is. Het fenotype van de *twt1-D* mutant en het expressiepatroon geven aan dat het gen mogelijk betrokken is bij de ontwikkeling van de vaatbundels.

Hoofdstuk 8 bevat een algemene discussie van het werk dat beschreven is in dit proefschrift. De verkregen resultaten worden met elkaar vergeleken en een algemene beschouwing over de bijdrage aan onze kennis van de plantontwikkeling is gegeven.

RESUMO

Estudos sobre a arquitetura das plantas não são importantes somente para a classificação botânica, mas também por razões agronômicas. Por exemplo, a arquitetura da planta influencia características do cultivo, aumentando a produtividade ou a qualidade do produto. Dessa forma, um melhor entendimento da arquitetura da planta consequentemente pode levar ao controle e a mudanças em características agronomicamente importantes.

A *Arabidopsis thaliana* é uma planta invasora usada como modelo para o entendimento biológico em espécies vegetais. A escolha dessa espécie se deve a suas inúmeras vantagens como genoma reduzido, curto ciclo vegetativo, número abundante de sementes, fácil transformação genética, e por ter tido recentemente seu genoma seqüenciado.

A técnica de "activation tagging", ou ativação gênica marcada, é uma poderosa ferramenta na identificação de novos mutantes, surgindo como uma alternativa na análise funcional de genes ao induzir mutantes com "ganho de função". Essa técnica é baseada no emprego de 4 cópias de elementos gênicos ativadores do vírus *CaMV* ("Cauliflower mosaic virus") *35S*, também denominados "enhancers".

Esta tese relata o estudo molecular no controle da arquitetura da planta, utilizando mutantes obtidos pela técnica de "activation tagging" em *Arabidopsis thaliana*.

O **capítulo 1** desta tese apresenta alguns conceitos e ferramentas utilizadas na obtenção de mutantes em *Arabidopis thaliana* que auxiliam no entendimento da arquitetura da planta; resume a pesquisa realizada com esta espécie modelo e relata as técnicas utilizadas neste estudo. O capítulo também explica os objetivos dos estudos realizados e descreve de forma sucinta os demais capítulos presentes nesta tese.

O **capítulo 2** mostra uma ampla revisão bibliográfica em que foram abordados importantes reguladores moleculares envolvidos em várias etapas do desenvolvimento da planta. Vários exemplos de ação antagônica entre genes são relatados e discutidos, auxiliando na compreensão de como as várias etapas do ciclo biológico vegetal são controladas nos níveis molecular e genético.

A ativação gênica em tecidos específicos é relatada no **capítulo 3**, onde se procurou gerar mutações induzidas em tecidos e órgãos pré-determinados. O emprego da ativação gênica em tecidos específicos pode superar efeitos não desejados gerados pelo uso dos "enhancers" do *CaMV35S*. Como exemplo desse tipo de estudo foi escolhido o "enhancer" do gene *SHATTERPROOF2* (*SHP2*). Esse gene está envolvido na formação na zona de deiscência do fruto de *Arabidopsis thaliana*, sendo expresso no pistilo e durante o desenvolvimento das síliquas. Seu "enhancer" foi ligado ao promotor do gene *FLORAL BINDING PROTEIN1* (*FBP1*) e ao promotor mínimo do *CaMV35S*. Como resultado, é demonstrado que o "enhancer" do *SHP2* em combinação com o promotor do *FBP1* ou do

CaMV35S dirige a expressão específica em tecidos da zona de deiscência do fruto de *Arabidopsis thaliana*, independentemente da distância ou da orientação em que o "enhancer" é ligado aos promotores. Isso indica que os "enhancers" podem ser usados em ativação gênica marcada para tecidos da zona de deiscência visando alcançar mutantes específicos nesse tecido, com intuito de interferir no processo de abertura das síliquas.

Entretanto, a ativação gênica marcada não possui somente vantagens. Uma grande desvantagem é relatada no **capítulo 4** desta tese. Nesse capítulo, investiga-se a possível causa para a baixa eficiência de ativação gênica marcada por meio de inserções de T-DNA. Enormes populações de plantas de *Arabidopsis thaliana* levando em seu genoma, o quádruplo "enhancer" do *CaMV35S*, são freqüentemente escrutinadas com o objetivo de encontrar mutantes morfológicos. Entretanto, a freqüência de fenótipos mutantes é baixa. Os resultados de experimentos relatados nesse capítulo indicam que existe correlação entre uma taxa mutacional baixa e a metilação dos "enhancers" do promotor *CaMV35S*. Todas as plantas contendo mais de uma inserção de T-DNA mostraram estar metiladas, com uma dramática queda de atividade do *CaMV35S*. Buscando-se uma alternativa a esse fenômeno, uma população de mutantes gerados por ativação gênica marcada por elementos transponíveis ("transposons") foi escrutinada, onde a taxa mutacional era 10 vezes maior do que em populações geradas por T-DNA. Com isso, três mutantes com alterações fenotípicas de interesse foram obtidos e analisados.

O **capítulo 5** relata os estudos realizados com o mutante *downwards siliques1* (*ds1-D*). Nesse mutante, os internódios são mais curtos do que o normal e os órgãos laterais, como as flores, estão direcionados para baixo, de aspecto semelhante ao mutante nulo *brevipedicellus* (*bp*). Estudos de expressão gênica demonstraram que esse fenótipo é devido a um ganho de função gerado pela superexpressão do gene *DS1*. Análises do mutante com perda de função do *DS1* não resultaram em um fenótipo alterado, sugerindo haver redundância com um outro gene. O gene mais próximo ao *DS1* é o *AS2*. Mutantes duplos *ds1 as2* foram gerados e, juntamente com outros resultados descritos nesse capítulo, foi possível concluir que esses dois membros da família LATERAL ORGAN BOUNDARY (LOB) agem de maneira redundante para controlar a determinação das células das pétalas de *Arabidopsis thaliana*.

Os estudos com um outro mutante, chamado *needle1-D* (*ndl1-D*), são descritos no **capítulo 6**. Essa também é uma mutação que gera um ganho de função no gene *NDL1*, um fator de transcrição da famíla "Helix-Loop-Helix (HLH)". A superexpressão do gene *NDL1* pelos "enhancers" do *CaMV35S* (que estão presentes no elemento transponível) alteraram a formação normal dos tecidos dos carpelos, nas síliquas de *Arabidopsis thaliana*. Assim, forma-se uma estrutura semelhante a uma agulha, no local da fusão dos dois carpelos, como em plantas do tipo selvagem. É interessante notar que o fenótipo apresentado no mutante *ndl1-D* assemelha-se a mutantes com perda de função que afetam

tanto o desenvolvimento do carpelo como o transporte de auxina, como o *ettin*, o *pinoid* e o *pin-formed*. A perda de função do gene *NDL1* não resultou, entretanto, em fenótipos visíveis, indicando que outros membros próximos na família dos "HLH" de fatores de transcrição podem estar agindo de maneira redundante. A expressão do gene *NDL1* é normalmente restrita ao pistilo e também às síliquas. Além disso, níveis de auxina no mutante *ndl1-D* são elevados, principalmente na estrutura alterada do gineceu, sugerindo que este gene tem atividade no transporte de auxina durante o desenvolvimento do carpelo em *Arabidopsis thaliana*.

O **capítulo 7** descreve os resultados dos estudos com o gene *TWISTED1* (*TWT1*), que foi identificado através do terceiro mutante *twisted1-D,* também um mutante com ganho de função. Nesse mutante, todos os órgãos são retorcidos, com um fenótipo mais acentuado nas síliquas, em função da superexpressão do gene *TWT1*. Análises da expressão gênica revelaram que esse gene é expresso no sistema vascular da planta *Arabidopsis thaliana*. De forma similar aos outros genes previamente descritos, *DS1* e *NDL1*, a perda de função do gene *TWT1* não acarreta em alguma mudança morfológica, sugerindo mais uma vez, redundância funcional. *TWT1* possivelmente transcreve uma proteína de membrana com função ainda desconhecida. Com o fenótipo do mutante *twt1-D* em combinação com as análises de expressão gênica do gene *TWT1* pode-se sugerir que esse gene participa no desenvolvimento de tecidos vasculares.

No **capítulo 8** é apresentada a discussão geral de todo o trabalho conduzido nesta tese. Os resultados de todos os capítulos estão conectados com uma visão geral de perspectiva de cada um para uma melhor compreensão da arquitetura e desenvolvimento das plantas.

ACKNOWLEDGMENTS

Yes, we got here! After all this period, sometimes it is hard to believe that we are here. Everybody has at this moment a feeling of success, mixed with a happiness that will never end. However, to get here, many people have been involved in these moments of success and sometimes, of sadness, who are a big part of this work. I have used "we" because I believe I would not be able to be here writing these words, without all of them. These words, my family and friends are as important to me as the entire experimental part of my thesis.

First of all, I must thank God for everything I have, not just during my time here, but during my entire life. Health, happiness, benignity, and especially, for giving me the two important people that I dedicate this work; You my beloved wife, Cristiane and my son Gabriel.

Secondly, I have to thanks my parents, Antonio and Djanira (*obrigado pai e mãe, por me ensinarem tudo que eu sei na vida e pude trazer comigo nesses momentos de criança, adolescente, jovem e adulto. Obrigado por me fazerem um "grande" homem. Obrigado por muitas vezes que você me disseram não e outras sim, e me mostrarem o caminho a ser seguido. Obrigado por terem me dado a educação que tenho e por me ensinarem a distinguir o certo do errado. Nunca esquecerei o quanto vocês trabalharam para nos fazerem as pessoas que temos sido. Talvez eu pudesse escrever um livro de tudo o que me ensinaram, mas esse livro é minha vida. Eu amo muito vocês!*). On my parents's side, I have my brother, who has been for quite a long time, my parent's support in my absence (*Gustavo! Muito obrigado por estar ao lado do pai, da mãe e da Ana por todo esse tempo. Você é uma pessoal muito especial, como irmão, filho, e como amigo e também como profissional. Eu amo você!*). In addition, to my beloved sister Ana Maria. (*"The Big", sim você é a maior! Muito obrigado pelo seu apoio em todos os momentos. Você é muito especial. Eu amo você minha querida irmã!*).

Moreover, I could not leave out the ones that were and are important to me. Especially, I would like to start with my Grandmother, "Vó Eti", who has been supporting me all this time (*Vó, muito obrigado por tudo que vc fez e tem feito por nós. Tenho certeza que todo esse tempo que estive fora, você sempre rezou por nós e pensou cada momento nas suas manhãs em nós. Eu amo você!*). On her side, it has been my aunt, who is a very important person (*Obrigado Tia Vera, você foi importante em todos os momentos da minha vida. Eu amo você!*). And definitely, I would never have been here without my unforgettable grandfather "Vovô Nagib". Unfortunately, you are not here anymore to see another important moment in my life. But I know that from whatever you are at this moment, you are happy for me, and even more, I know that you have always been looking after me (*Vovô, eu amo você!*). I have to state my gratitude and admiration to a person who

has been guiding me during all my professional and why not, personal life. Besides being my supervisor during my undergraduate studies, he was more than that, he was my uncle who always encouraged me to come abroad. Thanks Professor Nilton Nagib, and/or "Tio" Nilton. (*Muito obrigado por ensinar-me o caminho da ciência, por mostra-me o lado lindo dela e de como proceder na pesquisa. Aprendi muito com você. Você me direcionou a estar aqui hoje aqui neste momento. Muito obrigado de coração!*). My grandparents, Silvio (*in memoriam*) and Maria, I give my eternal acknowledgment (*Vocês sempre estarão em meu coração*).

I cannot forget the help from all our relatives. Thanks to all of you for supporting my wife and I (and now our baby) in those moments. We will never forget anyone (*Obrigado a vocês todos, tantos tios, tias, primos, primas, sobrinho e também amigos!*). Together with them, I want to express my gratitude to all my big "-in-law" family, mainly to my father-in-law Antonio, and mother-in-law Maria Lúcia (*Dona Maria Lúcia, muito obrigado por sempre acreditar em nós e pela eterna força que sempre nos deu. Compartilhe esse momento conosco!).* Amongst all our friends in Brazil, who have been supporting us for a long time, I want to give a warm thanks to the Versiani family (*Obrigado pelo apoio de vocês durante todo nossa jornada!*).

Before my arrival here in Wageningen, I had strong help from two persons: prof. Renato Paiva, who gave me suggestions and ideas for coming here. Thanks for giving me important tips and for saying: "if you want to go, you go. It depends on you". Dr. Marcelo Murad helped a lot with the proposal to come abroad. I will never forget the help I received from both of you. My thanks also goes to prof. José da Cruz Machado, the responsible person at the International Office at UFLA together with prof. Fabiano Ribeiro do Valle, the rector. In addition, my thanks to prof. João Bosco, at the department of Genetics at UFLA, for suggesting to me the contact (my future supervisor) here in Wageningen.

Here in Wageningen, at the Plant Research International (PRI, or CPRO when I got here), I met my supervisor prof. Dr. Gerco Angenent. Gerco thanks for accepting me into your lab to perform my Ph.D and for all the time we spent together. You are a great scientist and I have learned a lot from you. Thanks for your guidance during these 4 years, for helping me with everything (and I mean everything), for improving my chapters, and for financing part of my last 6 months here. In Gerco's lab, my thanks for everything to his crew: John, Marco, Jaqueline, Jeroen, Richard, Silvia, Stefano, Isabella, Ruud; former members Ana, Olga, Diana, Gaby, Esther, Tinneke, Yaxin and students. Thanks Faye for your comments and remarks on this thesis, and for correcting me with my misspellings, pronunciation and with "slangs". Moreover, I have to express my gratitude to Stefan de Folter. Thanks Stefan for showing me so many things at the beginning and I must not forget, for all the translations (*Bedankt menner!*). My thesis would not be the same without a very important person: Dr. Mark Aarts. Dear Mark, I really learnt a lot from you while

we were working together at PRI, and even now that you are at Wageningen University. You have made a substantial improvement in my career and I will never forget this (*Dank u wel!*). On my way to getting my degree, I have met another person: Jurriaan Mes. Besides sharing a room, you were later my diary lab supervisor from whom I have learned a lot. Thanks for all! I must thank our Business Unit members and our secretary Jannie Kramp, an excellent person and professional. You keep this Business Unit running. Still, more important people at PRI who have helped me to make the thesis concrete: Jan and Peter (Helpdesk at "de Haaff") and Ton van de Zalm (Helpdesk at "Born Zuid"); and so many others who indirectly or directly have given a hand to build this work. Thanks also to Andy Pereira and his group, Rafaella and Asaph for everything and especially Nayelli (*muchas gracias!*).

At the University, I had the enormous pleasure to know the outstanding prof. Maarten Koornneef, my promotor. Thanks Maarten for having me as your student. Even though we have not worked together daily, your contributions to this thesis were essentials. Thanks also for the funds for the last 6 months.

Working on a Ph.D. abroad is not easy. However, when you have friends it becomes nicer and easier. When we arrived here, we found a strong and exceptional Brazilian community, the big family, which we had so many unforgettable moments sprinkled with lots of "Grolschs". A few have returned to our unforgettable country and others are still here preparing to return. Of those who have left, I would like to thank, first of all, the Lage family. Thanks Gilberto ("Gigi le Grand"), Lucinha, Dudu e Vivi. You were more than friends to us. You were our second family during the time we spent together here in Wageningen, and I am sure that our friendship will never end (*Obrigado pela ajuda nos momentos mais difíceis de nossas vidas quando chegamos aqui em Wageningen, pelas piadas e pelo companheirismo, enfim por tudo!*); Alan "Captain", Ivete, Diego and now Mariana, who we have not yet known (*Valeu por tudo e por todos os momentos juntos que passamos!*); in the same way we discovered another special family: Paulo, Veridiana and Arthur (*Obrigado pela "força" que sempre nos deram, vocês também são três pessoas especiais!*); Luiz, Gilma, Guilherme and Cristiane (*Obrigadão por tudo que fizeram por nós!*); Amaral (*Eternamente: "tá gelada, tem problema?"*), Cláudia, Samuel and the new baby soon; an extraordinary couple, Eduardo (*"Grande" valeu por todas as partidas de squash e pela amizade!*) and Isabela; Vagner (*"Doutor Benedito", muito obrigado pelas discussões e pela força nesses anos juntos. Você é uma excelente pessoa!)*; Rômulo, Flávia and now Isabela (*Vocês são ótimos!*); Luiz ("Passarinho"), Simone, Alissa, Arielle and Jonas; Tarcísio, Franciene and Marina; Gustavo, Sandra, Pedro and Nina; Irene and Arne; Olavo Marineide and lovely girls Natália and Paola; Renato and Denise; Jorge and Flora; Raul, Viviane and children; Wellington, Denise and children; Rodrigo and Ana; Danny, Priscila and recently Lucca;

Flávia; Elaine; Elisa; Claudine and Sam; Brandão and Adriana; Fábio, Susan and Brenda; Débora; Jane and family; Pedro Boff, Mary and boys; Sara (*Um abraço para seus pais!*) and Graça (*Chicletes*). Those who are still making their life here in Wageningen, I wish you all the best. Francisco (*tudo de bom para você com muita sorte!*); Cristiano and Simone (*Valeu pelos jantares, "vinhadas" e risadas juntos. Vocês são muito legais!*); José Márcio, Regiane and Alissa (*"JM" capitão do nosso Ranca Toco Futebol e Regatas, continue firme e espero que você obtenha a "tríplice coroa" e cuidado senão só em 2010...*); Fausto and Tarsis; André (Raquel and Lucas); Isabella and Stefano (*Grazie amico!* You belong to the big nation now!); Celso; Marília; Joana; Mário and Ana Claúdia; Marta and "Meninas"; Milza; Renato.

I must mention that life here was not just the work. Thanks to Rene Kooyman and John Albers for our cozy Fridays at "de Bongerd" after our Brazilian squash duels. Thanks to the memorable friendship. To the members of our soccer teams during these years (not so many prizes, but lots of fun) and soccer recreation members at "de Bongerd". Thanks to everybody for the unforgettable moments and matches.

My special thanks to my Brazilian governmental sponsor, CAPES (Coordenação de Pessoal de Nível Superior) that financed me all these years and made my dreams come true. I would also like to thank the IAC (International Agriculture Centre, Wageningen), for partially funding the last 6 months of this work.

Finally, I have no words to express my feelings to you my wife Cristiane. You have been my support during all these years; you have always been pushing me towards our goal, which is this important moment in our lives. (*Meu amor! Saiba que essa tese nunca sairia se você não estivesse ao meu lado. Você abandonou tudo por esse momento e hoje estamos conseguindo juntos. Você é a dona desse sucesso! Eu te amo e sempre te amarei. Obrigado por ser essa pessoa maravilhosa e cheia de vida que você é, pelo seu carinho, paciência, compreensão, apoio, entusiasmo, pelo seu ouvido e sobretudo, pelo seu amor!*). As a reward after these 4 years in Wageningen, we got our angel Gabriel. (*Meu filho! Eu te amo e a você dedico minha vida e meu coração. Obrigado por ter vindo ao mundo em um momento tão especial e estar ao meu lado para festejarmos juntos!*).

Lastly, I have to say that finishing a Ph.D here in Wageningen is much more than earning a degree. It is gaining friends, constructing memories, and mainly learning how to build a better live. I will never forget these years here.

Thank you very much to all!

Obrigado!

THE AUTHOR

Antonio Chalfun Junior was born in Lavras, in the Southern region of the Minas Gerais, Brazil, on April the 17th, 1973. In this city, known as "Terra dos Ipês e das Escolas" (Land of "Ipê" (*Tabebuia spp)* and schools) in reference to all the "Ipê" trees and schools which exist there, he took his primary school studies and, in 1981, he moved with his family to Varginha, which is also in the Southern region of the Minas Gerais state. There he finished his primary and high school studies. Later, he returned to the city of his birth, for undergraduate studies in Agronomy at the "Universidade Federal de Lavras" (UFLA), where he graduated in 1996. During his undergraduate studies, he was awarded with fellowships from two governmental institutions: CNPq (Conselho Nacional de Desenvolvimento Cientíco e Tecnológtico) and CAPES (Coordenação de Aperfeiçoamento de Nível Superior), which stimulate the scientific career of undergraduate students. In 1996, at the same university, he started a M.Sc program on fruitculture, in the Agronomy Department, funded by CAPES. These studies resulted in a dissertation describing the work carried out on peach stones. The effects of different time and conditions of storing peach stones in the final production of peach rootstocks were analysed. In 1999, he received his degree and was granted by CAPES a 4-year-Ph.D. scholarship to go to the Netherlands to pursue his studies. On October the 26th, 1999 he arrived in Wageningen. He conducted his Ph.D. at Wageningen University and Research Centre, specifically at Plant Research International. The resulting thesis comprises the molecular analysis of plant architecture using activation tagging, which he completed on April the 21st.

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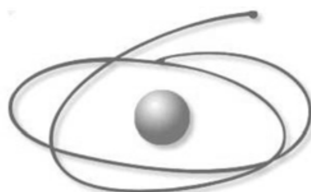
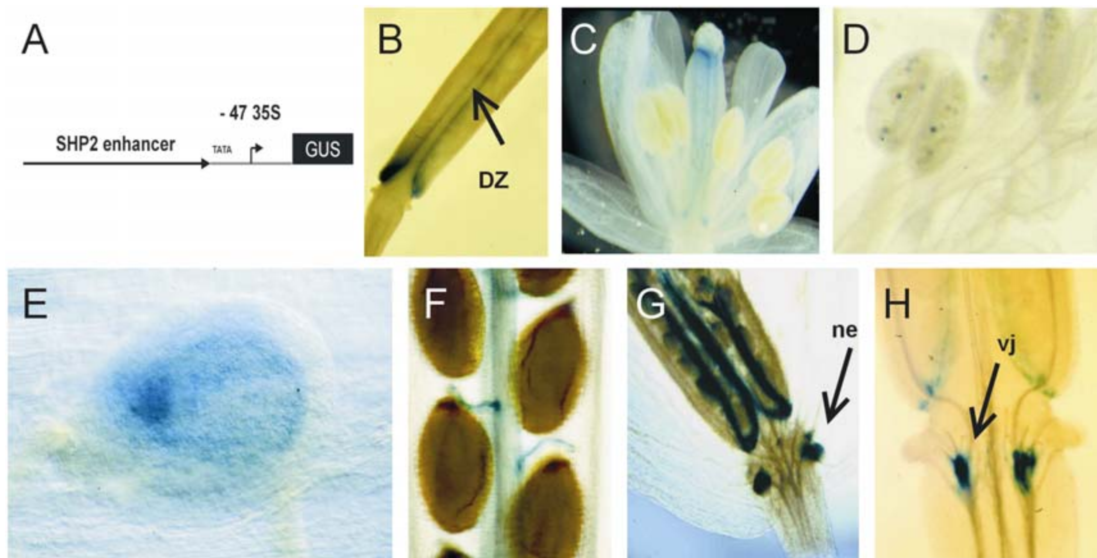
This work was financially supported by
CAPES (Coordenação de Aperfeiçoamento de Nível Superior), Brazil.
BEX 1519/98-0



APPENDIX
(Colour figures)



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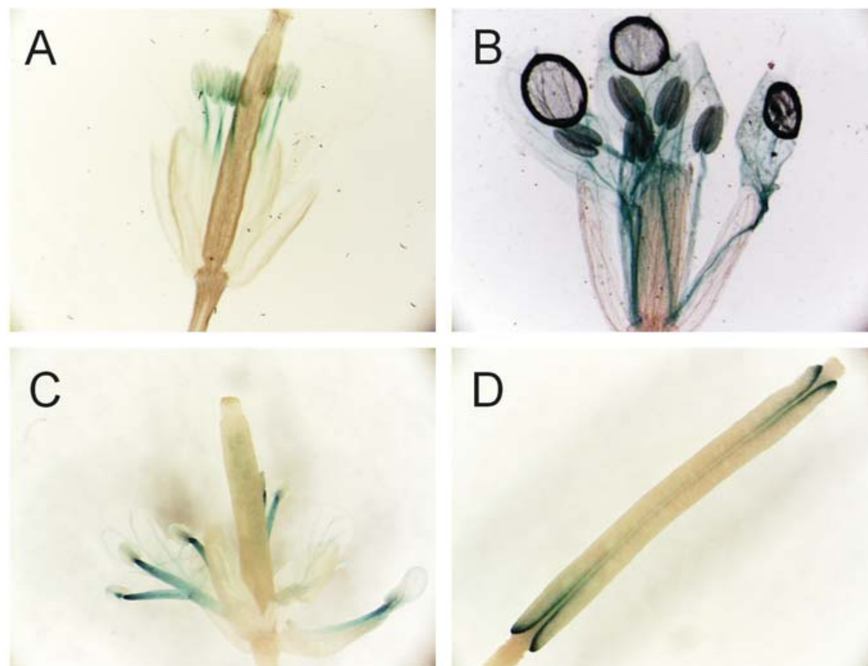

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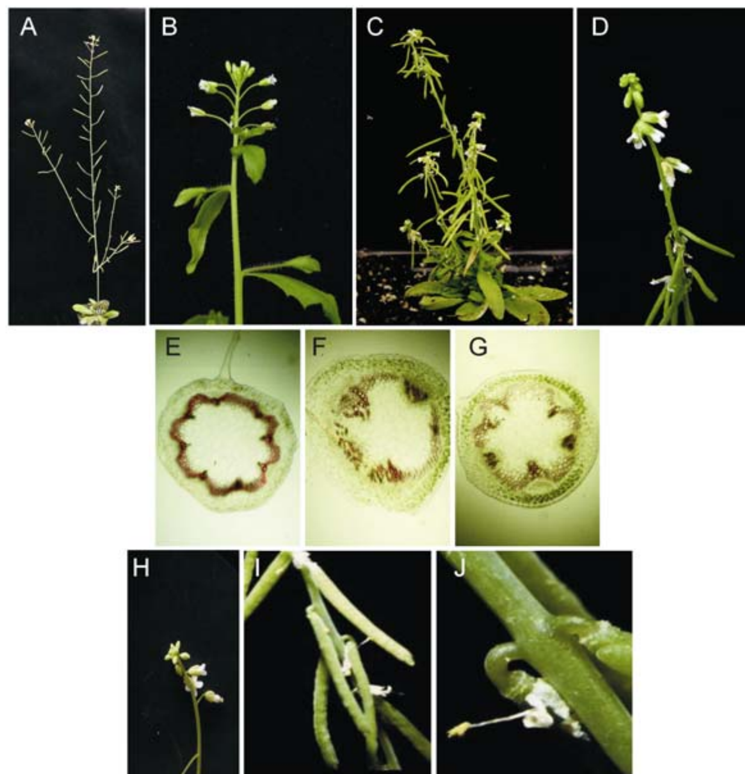

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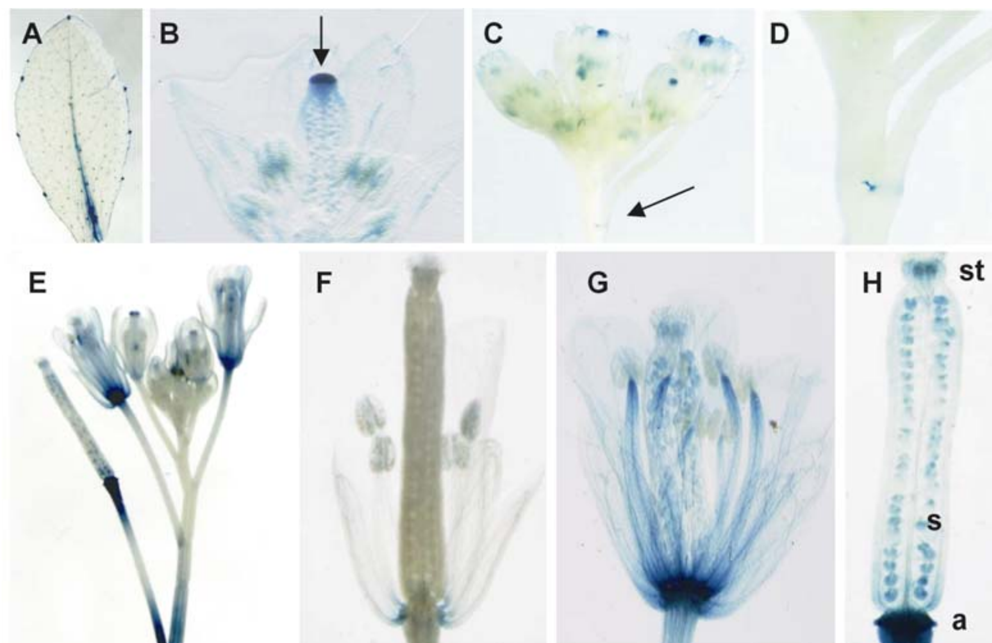
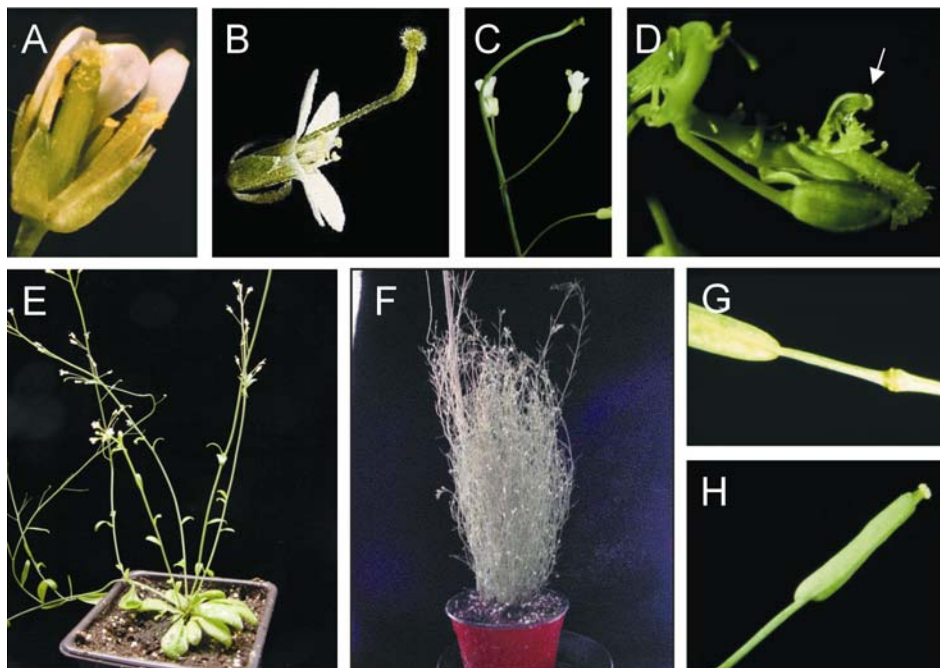


CHAPTER 5 - Figure 4

CHAPTER 5 - Figure 5



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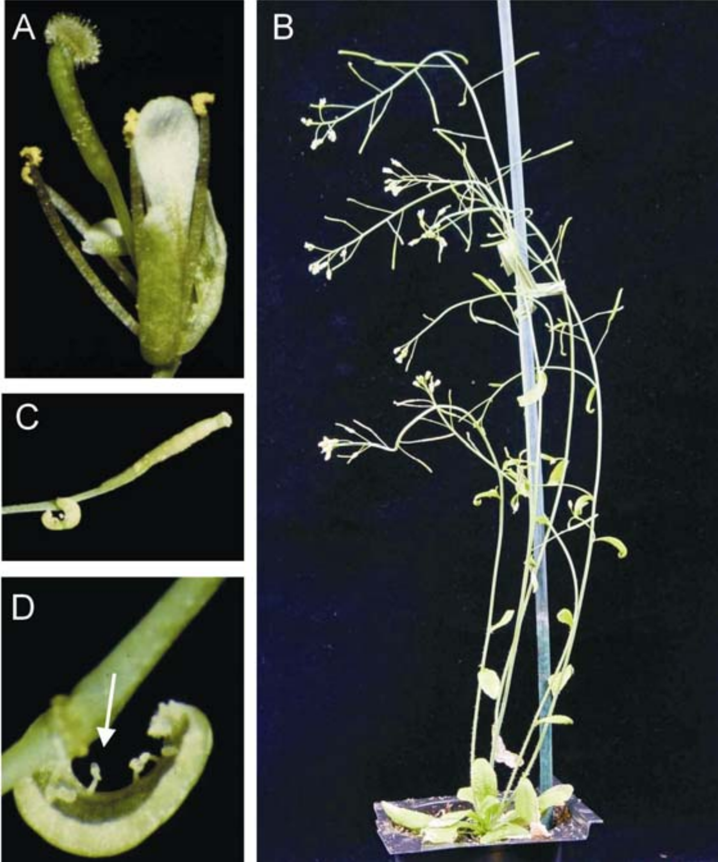

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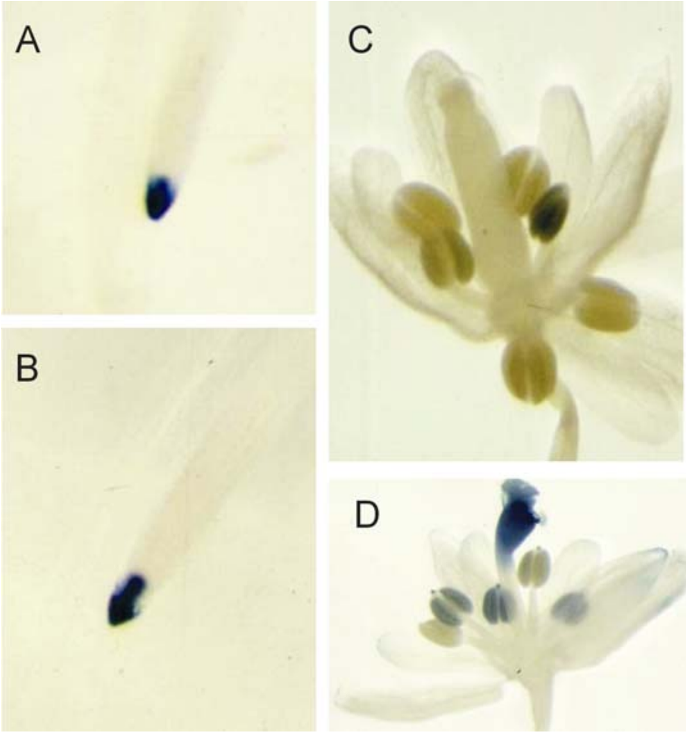

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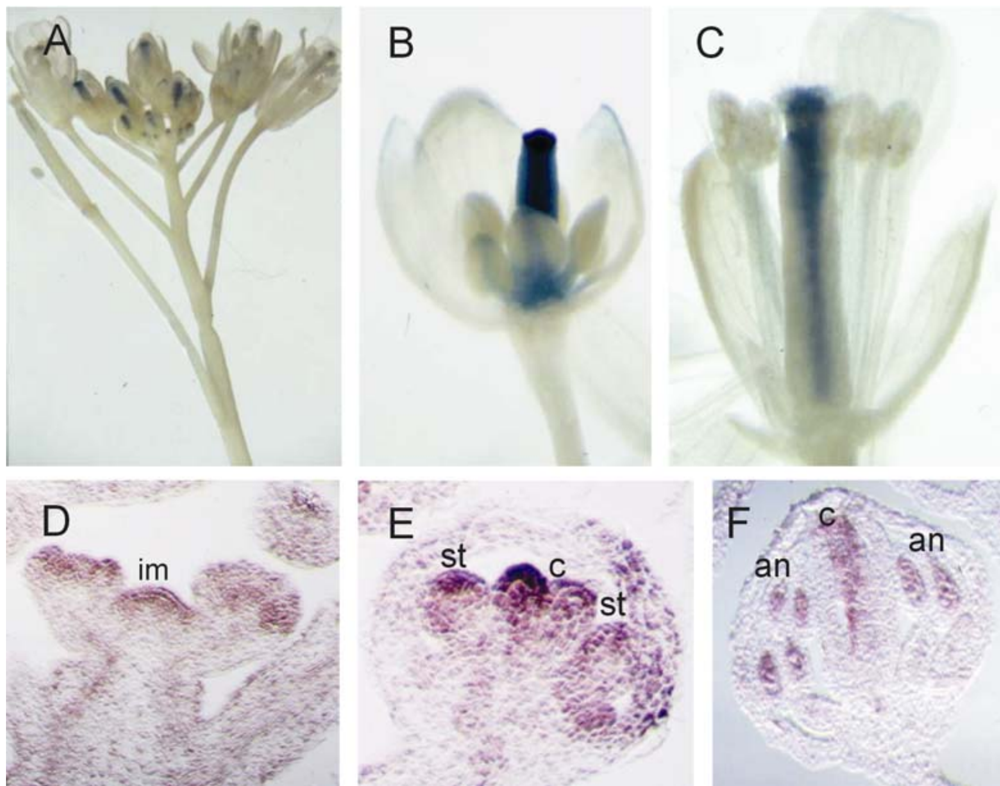
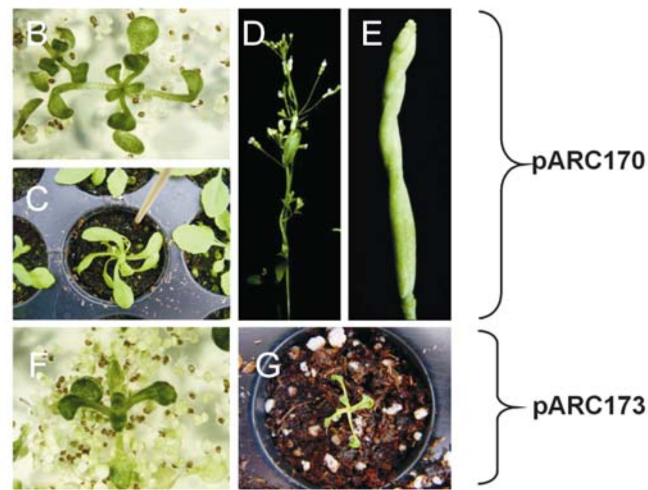
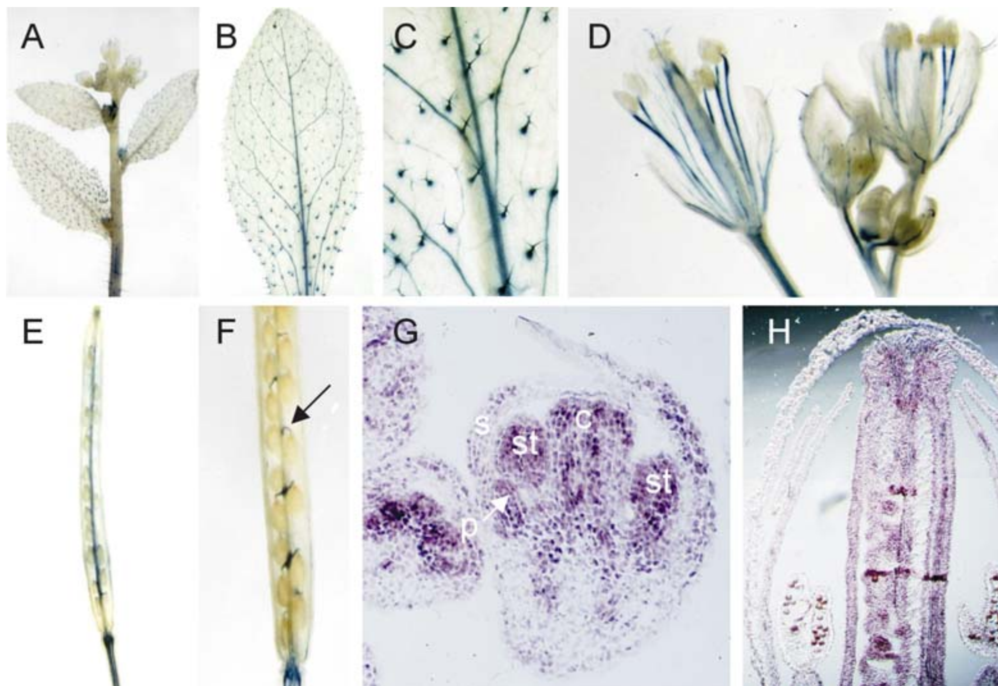


CHAPTER 6 - Figure 6

CHAPTER 7 - Figure 1



CHAPTER 7 - Figure 4



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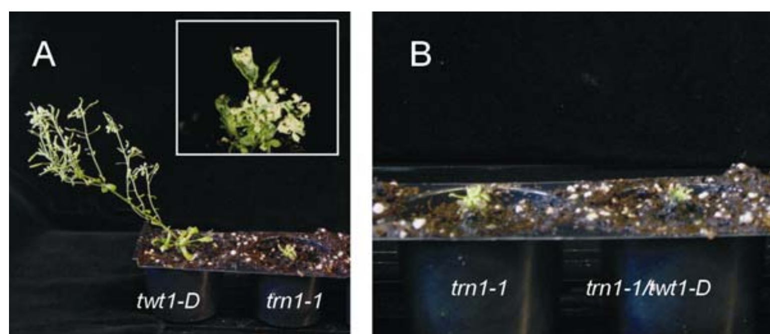

CHAPTER 7 - Figure 7