Flower development of *Lilium longiflorum*:

Characterisation of MADS-box transcription factors

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"To the people from Brazil,
I dedicate this thesis"

Por isso na impaciência
Desta sede de saber,
Como as aves do deserto
As almas buscam beber…

Oh! Bendito o que semeia
Livros… livros à mão cheia…
E manda o povo pensar!

O livro caindo n'alma
É germe - que faz a palma,
É chuva - que faz o mar.

Castro Alves,
Brazilian poet

CONTENTS

INTRODUCTION

Lilium longiflorum and its molecular floral development

Lily *(Lilium* spp.) is among the most traditional and beloved ornamental flowers worldwide. The genus *Lilium* comprises almost one hundred species, among which is the primary subject of our research, described in this thesis, the species *Lilium longiflorum* (Thunb.), known as trumpet lily or Easter lily.

Despite the great economic importance of ornamental lily species, little is known about its biology at the molecular level so far. In a time when two plant genomes are fully sequenced, *Arabidopsis thaliana* and *Oryza sativa*, only a few genes have been characterized in *Lilium* spp. Possible reasons for this are discussed below and throughout this work.

This thesis intends to be a contribution to bridging the fundamental research concerning transcription factors involved in development of flower morphology in model species and the applied objectives of molecular breeding for manipulating the flower morphology, endeavouring to create new cultivars with specific and novel features, more specifically in *Lilium* spp.

Lily Floral Development

Coordinated molecular interactions in lily lead to the development of beautiful organs called flowers. Lily flowers can reach up to 20 cm in length before anthesis. They are structured in concentric whorls. Six showy, petal-like organs form the first and second outermost whorls, consisting of three organs per whorl. These similar organs are denominated tepals, instead of the sepals and the petals found in the first and second whorls in model dicot species, which differ clearly in their appearance. Six stamens are displayed in two adjacent whorls (which were designated as only one whorl throughout this thesis for the sake of simplicity), and a pistil formed by three fused carpels is located in the innermost whorl.

Recently, Tzeng and Yang (2001) described the floral structure and development of *Lilium longiflorum*. In brief, a 2-mm floral bud already has its tepal, anther and filament structures formed whereas the carpel is not fully differentiated yet, being complete only later, in a 10-mm bud. A floral bud of 30 mm has all its organs well developed, with the pistil showing stigmatic papillae, distinct style, ovary and ovules. Anthesis occurs when the bud reaches around 200 mm in length.

Knowing the mechanisms of which coordinate molecular interactions to convert a vegetative meristem into a floral meristem and subsequent floral organ determination is the key for manipulating floral phenotypes. Many studies have been carried out on molecular development of flower morphology in model species. However, in lily this task began much later and is still in its infancy.

Working with lily in the lab

Transferring knowledge obtained with model species to crops with economic value is a tough task. Model species are established as such because they present methodological advantages over species of economic value, allowing to ease and to speed their investigation.

Lilium longiflorum is equipped with a massive genome size, calculated to be almost seven hundred times the *Arabidopsis* genome and about ten times the human genome. This leads to difficulties in applying molecular techniques. Southern blot analysis, which is a plug-and-play procedure for model species, is not applicable to lily in most instances (Kanno et al., 2003; this thesis), since it requires an enormous amount of DNA to be digested and loaded onto a gel in order to get a sufficient signal.

Northern blots are not dependent on genome size, but the isolation of total RNA to perform Northern blots also requires considerable efforts for finding the optimal method, possibly due to the high polysaccharide contents in lily tissues. RNA proved difficult to isolate from flowers using the traditional protocols based on phenol extraction followed by precipitation with lithium chloride.

Additionally, a lily transformation method mediated by *Agrobacterium* proved to be difficult, and the bombardment procedures still require great efforts in order to produce transformed plants.

Bulbous species also require a long generation time, since the first seasons after germinating are spent in bulb growth instead of sexual reproduction, which will come only some years later. This consumes a considerable time lag to know the inheritability of a given gene. Therefore, genetic studies are very time consuming in lily.

Nevertheless, the lily flower is a good model in other aspects, e.g., it has been used extensively for androecium development and biochemical pathway investigations (Kim et al., 1993; Wang et al., 1996; Mousavi et al., 1999; Poovaiah et al., 1999; Mori and Tanaka, 2000; Allwood et al., 2002; Ko et al., 2002; Singh et al., 2002), due to the pollen abundance and easy manipulation in consequence of its flower size.

Optimisation of the lily transformation system

An effective transformation procedure requires easy and fast transgene integration approaches combined with efficient regeneration methods in order to produce transgenic plants with a minimum of capital, energy and time input.

Tissue culture approaches for lily are well established already, with callus induction and plant regeneration at high efficiency and in a short time frame. In spite of that, there are currently a considerable number of studies aiming at improving the efficiency, especially with respect to clonal mass propagation and culture of cell types for higher transformation rates (reviewed by Aswath et al., 2001). Despite these beneficial tissue culture aspects, a lily transformation system is not very efficient yet.

The current lily transformation system that is available is based on particle bombardment, and only two groups have been able to produce transgenic lilies so far (van der Leede-Plegt et al., 1997; Watad et al, 1998). This transformation method is much more laborious, expensive, and variable than the biological systems. It has been demonstrated that a transformation system for lily based on *Agrobacterium tumefaciens* is feasible, since agroinfection in lily leaves was demonstrated earlier (Langeveld et al., 1995). We have also found transient GUS expression in infected lily leaves but we were unable to get transgenic plants using this approach. More recently, Mercuri et al. (2003) presented the first transgenic lilies derived from *Agrobacterium*-based transformations. Studies are also being carried on in our group to investigate the potential and feasibility of *Agrobacterium*-mediated transformation system in *Lilium longiflorum*.

The MADS-box genes

MADS-box genes constitute a family of transcription factors involved in cell development of a variety of eukaryotic life forms, from yeast to plants and animals. MADS is an acronym to the first four members which founded this family: *MINICHROMOSOME MAINTENANCE 1* (*MCM1*) from yeast, *AGAMOUS* (*AG*) from *Arabidopsis*, *DEFICIENS* (*DEF*) from *Antirrhinum*, and *SERUM RESPONSE FACTOR* (*SRF*) from human (Schwarz-Sommer et al., 1990; Yanofsky et al., 1990).

Members of this family have a highly conserved motif of about 56 amino acids called MADS domain that, in order to activate the transcription processes, binds to CArG *cis*-elements ($CC(A/T)_6GG$) found in target gene promoters (Hayes et al., 1988; Riechmann et al., 1996). Whereas the MADS domain is responsible for binding to specific regions of the DNA, other portions of the protein are involved in protein-

protein interactions and activation of the molecular transcription machinery (Treisman, 1990).

MADS-box genes are involved in many aspects of plant development, including meristem identity, flowering time, flower determination, floral organ identity, pollen fertility, ovule development, fruit identity and development (Causier et al., 2002), and lateral root elongation (Zhang and Forde, 1998). Expression of MADS-box genes was also found in leaf guard cells and trichomes (Alvarez-Buylla et al., 2000). This gene family encompasses 107 members in the *Arabidopsis* genome (Parenicová et al., 2003).

Plant MADS-domain proteins are divided primarily in two main types (Alvarez-Buylla et al., 2000b). The type I proteins are structurally similar to MADS-domain proteins found in other kingdoms, with the very conserved MADS domain and a specific carboxy terminal extension. There are 68 type I MADS-box genes in the *Arabidopsis* genome. They have been subdivided into five subfamilies designated Mα (with 25 genes), Mβ (20 genes), Mγ (16 genes), Mδ (6 genes), and the secluded *AGL33* (Parenicová et al., 2003). Despite encompassing 63% of MADS-box genes in *Arabidopsis*, functionality of the type I genes has not been easy to assign. This fact has changed only recently, when Köhler et al. (2003) demonstrated that *PHERES1* (*PHE1*), formerly *AGL37* (Mγ subfamily), is involved in seed development. However, reasons for its active expression in leaves (Parenicová et al., 2003) are still unexplained.

Type II MADS-domain proteins are found in the plant kingdom and are characterized by a MIKC structure (Purugganan et al., 1995), which implies a highly conserved MADS domain (M), involved in DNA-binding, a non-conserved intervening region (I) linking to the conserved keratin-like domain (K) responsible for protein-protein interactions, followed by a weakly conserved carboxy-terminal (C) portion that is involved in transcriptional activation. In some MADS proteins, an amino-terminal portion (N) is also found before the MADS domain, although it seems to have no activity, since proteins with a truncated N-terminus remain functional. There are 39 type II MADS-box genes in *Arabidopsis*, of which 17 were thoroughly characterized with loss-of-function mutants (Parenicová et al., 2003).

Flower organ development and the ABCDE model

A typical dicotyledoneous flower is arranged in four concentric whorls with defined organs (sepals, petals, stamens and carpels from outer to innermost positions). Having varied greatly their floral organ shapes and inflorescence architectures

throughout evolution, plant species inspired Carl Linnaeus in the 18th century to propose his *Systema Naturae* binomial classification based mostly on flower morphology.

In the same 18th century, Johan Wolfgang von Goethe, based on anatomical studies of plant development, proposed that every floral organ was in fact a modified leaf (1790).

However, it was not before the early 1990s that a molecular model was formulated that could explain the metamorphosis of leaves into floral organs. The ABC model for flower development, conceived by Coen and Meyerowitz (1991) is an elegant and neat model, which they poetically referred to as "the war of the whorls".

This model was described as the expression of overlapping genetic functions in two adjacent whorls, leading to the composition of the different floral organs. When only type A genes are expressed, sepals are generated; when type A plus type B genes are expressed, petals arise; concomitant expression of type B and type C genes leads to stamen formation; and when only type C genes are expressed, carpels are developed. Additionally, type A and C genes are antagonists, with the A domain in the two outermost whorls and the C domain in the two innermost whorls.

Another important aspect is that the C functional gene is also responsible for the determinacy of the flower and, when the C function is not active, a new flower emerges in the fourth whorl in a reiterated fashion. The model itself does not explain this characteristic but, since this function was revealed by C-type mutants, the determinacy loss of the flower was adopted.

A D function was added to the early ABC model as a result of studies of homeotic MADS-box genes involved in *Petunia* ovule development (Angenent et al., 1995; Angenent and Colombo, 1996; Colombo et al., 1995), making this species another model for flower development at a genetic and molecular level (Immink et al., 1999; Immink and Angenent, 2002; Kapoor et al., 2002).

Two hundred years after the insightful proposition of Goethe declaring that the ground state of every floral organ is a vegetative leaf, Bowman et al. (1991) could predict and produce a triple ABC-type mutant in which each floral organ resembled the ground state, a cauline-like leaf. Conversely, a leaf could not be transformed into a floral organ with the genetic toolkit available at that time, since the ABC genes were recognisably necessary but not sufficient for this homeotic conversion.

The question could only be answered with the discovery of the *SEPALLATA* function. The production of a *sepallata* (*sep*) *1*, *2* and *3* triple mutant led to reach the ground state (sepal-like organs in each whorl) even when the ABC genes were

functionally active (Pelaz et al., 2000). Moreover, the metamorphosis of leaves into petals is proved possible now, when a *SEP* gene is ectopically expressed together with the other A and B genes (Honma and Goto, 2001; Pelaz et al., 2001). This confirms clearly the hypothesis that a petal is a modified leaf. The *SEP* function was then recognised as the mysterious missing factor to the homeotic conversion of a leaf into a petal, being added to the model for flower development as the E function, designating the model as ABCDE from then on (Theissen, 2001).

Despite the attention given to the ABCDE genetic regulation, little is currently known about the events downstream the ABCDE model towards the organ set up (Sablowski and Meyerowitz, 1998). The identification of the immediate targets of the transcription factors of the ABCDE model is a *sine qua non* for understanding the genetic mechanisms involved in floral organ formation. High throughput methodologies have been applied recently in the quest to identify genes that participate downstream the ABCDE genes in order to promote floral organ development (Scutt et al., 2003; Zik and Irish, 2003).

The ABCDE model for flower development in dicot and monocots

The early ABC model for flower development was assembled based upon the genetic configuration of two dicot species: *Arabidopsis thaliana* and *Antirrhinum majus* (Coen and Meyerowitz, 1991). The model proved to be very conserved among dicot species and was readily accepted by the scientific community. However, the ABC model could not be directly applied to monocot species, since there are major divergences in their floral organ anatomy and architecture that do not support the model as in its first conception.

The species belonging to the Poaceae family show flowers with substantial anatomical differences in their perianth organs, with palea, lemma and lodicules, instead of sepals and petals. There is still a great debate to evolutionarily correlate these organs to those found in dicot species (Kramer and Irish, 2000; Kyozuka et al., 2000).

Another monocot family with contrasting characteristics in the flowers is the Liliaceae. The most notable difference found in this family is that the flowers do not have a perianth formed by distinguishable organs, sepals and petals, but, instead, they have two whorls formed by identical organs, designated tepals. Adaptation to the ABCDE model was not difficult, since an extension of the B function to the first whorl could lead to this feature. A phenotype that corroborated with this hypothesis was

found in the *Viridiflora* tulip mutant, in which sepals were observed in the place of tepals, probably due to the loss of its B function (van Tunen and Angenent, 1993).

More recently, a B functional gene from *Lilium longiflorum*, *LMADS1* which is an *AP3*/*DEF* orthologue, was accessed by molecular analysis, showing expression activity in both perianth whorls (Tzeng and Yang, 2001). Its correspondent protein, however, was not detected in those tepals derived from the first whorl.

In eudicot species, B type proteins must form heterodimers in order to be stable and functionally active (Goto and Meyerowitz, 1994; Yang et al., 2003). Results obtained for the corresponding monocot orthologues from maize suggested similar heterodimer stabilisation (Ambrose et al., 2000). Nevertheless, experiments using putative orthologues from *Lilium regale* confirmed that its B type proteins could indeed form heterodimers, but, surprisingly, the *L. regale PI*/*GLO* putative orthologue was also stable *in vitro* as homodimer, whereas the *L. regale AP3*/*DEF* putative orthologue behaved as a typical dicot counterpart, i.e. only being able to form heterodimers (Winter et al., 2002). These results are also observed in the respective orthologues from tulip (Kanno et al., 2003). It may be possible that the *PI*/*GLO* homologues in the Liliaceae family can act alone in the first whorl to induce the homeotic identity change of sepals into petals. This assumption, however, still requires further investigation using *in vivo* procedures.

Heterologous genetic characterisation in model species

Despite the differences found in the ABCDE model between dicot and monocot species and the huge flower variability among the Angiospermae species, the model shows consistency throughout evolution.

As stated before, the ABCDE model were established using the model species *Arabidopsis* and *Antirrhinum*, and later, *Petunia*. In monocot species, such as rice and maize, many MADS-box genes have been studied, but the complexity involved in the floral anatomy of these species creates difficulties to set up a definitive and general ABCDE model for monocots so far.

However, despite the dicot-monocot evolutionary divergence that happened circa 120-180 million years ago (Wolfe et al., 1989), MADS-domain proteins derived from both classes are still able to cross interact in yeast in a proper fashion (Favaro et al., 2002), indicating the importance of their functions throughout evolution.

The evolutionary strength of the ABCDE model for flower development facilitates functional studies of orthologous genes, allowing using model species as

heterologous systems. This circumvents the analysis of the species of interest that usually present significant drawbacks, such as lack of null phenotypes for the genes in study, long generation time, difficulties to generate transgenic plants and so on.

Arabidopsis has been by far the most used heterologous system in developmental biology. Many species have their MADS-box genes functionally characterized in *Arabidopsis* by complementing mutants or overexpressing heterologous genes, or producing dominant negative phenotypes. Examples of heterologous characterisation of MADS-box genes can be found with genes derived from lily (Tzeng and Yang, 2001; Tzeng et al., 2002), hyacinth (Li et al., 2002), lisianthus (Tzeng et al., 2002) and orchid (Hsu and Yang, 2002), to name just some ornamentals.

A new possibility has emerged

In the genomics era, the concept of a good model species does not merely lie in the ease of manipulation. Now, there is also a requirement for high throughput data production. In the case of floral studies, escaping genetic transformation is highly desired, since it demands a generation time from transgene insertion to phenotype evaluation.

To supply this current demand, a new system has emerged, promising easy technical manipulation and speedy results. The virus-induced gene silencing (VIGS) system (Baulcombe, 1999), based on post-transcriptional gene silencing (PTGS) mechanisms (Ratcliff et al., 1997; Baulcombe, 2002; Tang et al., 2003), is being claimed as the most promising technology of the future for functional genetic characterisation.

Thus far, the system is working efficiently in the model species *Nicotiana benthamiana* (Burton et al., 2000), but some restrictions are still imposed to other species, such as *Arabidopsis* and lily. Tomato (Liu et al., 2002a) and rice (Holzberg et al., 2002; Kanno et al., 2000) are coming up as workable systems among the economically important species.

Many new viral vectors have been engineered (Kumagai et al., 1995; Ruiz et al., 1998; Ratcliff et al., 2001; Peele et al., 2001; Gosselé et al., 2002; Holzberg et al., 2002; Turnage et al., 2002), allowing the system to cover more plant species. There is also an increasing number of genes with their function elucidated by this method (Burton et al., 2000; Liu et al., 2002b), and the genetic and molecular mechanisms on which the system is based, is being gradually unveiled (reviewed by Tijsterman et al., 2002; and this thesis).

Lily challenges the floral development model one more time

After the establishment of the early ABC model as a general model for flower development, the Liliaceae family challenged the model for the first time, since its flowers do not show clear distinction among the organs in the first and second whorls. The problem was solved by proposing a modified ABC model for Liliaceae members, in which the B function is extended to the first whorl (van Tunen and Angenent, 1993; Theissen et al., 2000). The current modified ABCDE model for Liliaceae has shown to be suitable, at least up to a certain extent, and a genetic analysis of C and E functions is reported in this thesis.

Surprisingly, we recently found a natural lily mutant with a complete homeotic change in only one floral organ type: each stamen turned into a tepal, whereas its pistil is completely normal. We named this mutant *festiva* and there is no report of a homologous mutation in *Arabidopsis*.

One can speculate that the mutation involves genetic regulation up or downstream the ABCDE model but a different operation of the model itself, with an additional factor, cannot be ruled out.

SCOPE OF THE THESIS

To investigate the molecular basis of the ABCDE model in *Lilium longiflorum*, we characterized a putative *AGAMOUS* (*AG*) orthologue (*LLAG1*) in chapter two, demonstrating that *LLAG1* heterologous expression induces homeotic changes, which indicates a function similar to *AG*. Moreover, a double flower lily mutant, reminiscent of the C null *Arabidopsis* mutant, *agamous*, is also discussed.

The putative lily E orthologue, designated *LLSEP3*, is characterized in chapter three of this thesis, suggesting that the E function is present in Liliaceae members, and showing once more the strength of the ABCDE model throughout evolution.

Chapter four reports the drawbacks and difficulties encountered when using *Arabidopsis* for a functional analysis of a lily gene, in spite of the general assumption that working with model species is a “plug-and-play” task.

Transformation of *Lilium longiflorum* via particle bombardment and the recovery of herbicide-resistant transgenic plants are reported in chapter five.

Although the *festiva* natural lily mutant arose too late for detailed molecular investigation here, we speculate on the possible mechanisms underlying such phenotype in chapter six, as well as referring to a double, *agamous*-like lily mutant.

In chapter seven, we raise the possibility of using the VIGS system to functionally analyse MADS-box genes, including those belonging to the flower development model. A general presentation of the system, including its mechanisms, and our preliminary results using a lily gene in *Nicotiana benthamiana* are also included in this chapter.

In the last chapter of this thesis we present a general discussion of our data, some hypotheses and the perspectives for future investigation on lily flower development.

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CHAPTER 2

Ectopic expression of *LLAG1*, an *AGAMOUS* homologue from lily (*Lilium longiflorum* Thunb.) causes floral homeotic modifications in *Arabidopsis*

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Abstract

The ABC model for floral development was proposed more than 10 years ago and since then many studies have been performed in model species, such as *Arabidopsis thaliana*, *Antirrhinum majus* and many other species in order to confirm this model. This has led to additional information on flower development and to more complex molecular models. *AGAMOUS* (*AG*) is the only C type gene in *Arabidopsis* and it is responsible for stamen and carpel development as well as floral determinacy. *LLAG1*, a putative *AG* orthologue from lily (*Lilium longiflorum* Thunb.) was isolated by screening a cDNA library derived from developing floral buds. The deduced amino acid sequence revealed the MIKC structure and a high homology in the MADS-box among *AG* and other orthologues. Phylogenetic analysis indicated close relationship between *LLAG1* and *AG* orthologues from monocot species. Spatial expression data showed *LLAG1* transcripts exclusively in stamens and carpels, constituting the C domain of the ABC model. Functional analysis was carried out in *Arabidopsis* by overexpression of *LLAG1* driven by the CaMV 35S promoter. Transformed plants showed homeotic changes in the two outer floral whorls with some plants having the second whorl completely converted into stamens. Altogether, these data indicate a functional relationship between *LLAG1* and *AG*.

Introduction

Mechanisms of flower development are under control of a complex genetic system. The formation of organs in the four whorls of a typical eudicotyledonous flower, consisting of sepals, petals, stamens and carpels, requires many genes for proper organ and tissue development (Blázquez, 2000).

Transcription factors play major roles in the genetic regulation, being responsible for orchestrating the cascade of processes for cellular development, differentiation and maintenance. Homeotic genes encode transcription factors involved in the specification of organ identity; their loss of function results in the replacement of one type of organ by another.

Several studies with *Arabidopsis thaliana* and *Antirrhinum majus* led to the so-called ABC model of flower development (Coen and Meyerowitz, 1991). This model postulates that three classes of homeotic genes control floral organ formation in a combinatorial fashion. Expression of type A genes alone, A plus B, B plus C and C alone triggers the formation of sepal, petal, stamen and carpel, respectively. In addition, A- and C-functions are considered antagonists since activity of one type represses the activity of the other (Bowman et al., 1991).

The ABC model of flower development has been fine-tuned in time. Important extensions in the model were the addition of a D function for ovule development (Angenent et al., 1995) and, more recently, the inclusion of an E function of which transcription is active in the inner three whorls of the flower. Together with other functional gene classes, the E function is indispensable for the development of petals, stamens and carpels and contributes to protein complex formation in order to trigger proper differentiation of those organs (Pelaz et al., 2000, 2001; Honma and Goto, 2001). New proposals for transcriptional protein interaction, among others the "quartet model" which postulates the multimeric complex formation of MADS box proteins and DNA regions for different organ development in the floral whorls also arose recently due to the forementioned discoveries on the ABC model, hence called ABCDE model (Theissen and Saedler, 2001; Honma and Goto, 2001).

All the genes involved in the ABCDE model are MADS-box genes, with the exception of the type A *AP2* gene. The MADS-box genes constitute a superfamily of transcription factors found in very simple organisms e.g. yeast as well as in complex species, such as plants and animals (Schwarz-Sommer et al., 1990; Shore and Sharrocks, 1995; Ng and Yanofsky, 2001). In plants, they are involved in many developmental processes, especially in the reproductive organs (Ng and Yanofsky, 2001), but also in roots, leaves and other organs (Alvarez-Buylla et al., 2000; Causier

et al., 2002). Plant type II MADS-box proteins carry a conserved organisation called MIKC, which is characterised by the highly conserved DNA-binding motif of 56 amino acids called MADS-box (M). The weakly conserved intervening (I) region is thought to be the determinant factor of selective dimerization. The moderately conserved K-domain, with its keratin-like coiled-coil structure is involved in protein-protein interactions and is exclusively found in plant species. Finally, the variable carboxy-terminal (C) domain may be responsible for transcriptional activation and protein interaction stabilisation (Egea-Cortines et al., 1999; Riechmann and Meyerowitz, 1997).

AGAMOUS (*AG*) is the only C-functional gene found in *Arabidopsis,* whilst in other species redundant or complementary *AG* paralogues have been found, such as *PLENA* (*PLE*) and *FARINELLI* (*FAR*) in *Antirrhinum majus* (Davies et al., 1999), *FLORAL BINDING PROTEIN 6* (*FBP6*) and *FBP14* (*pMADS3*) in *Petunia hybrida* (Kater et al., 1998). In *Arabidopsis*, recessive mutants for *AG* show petals instead of stamens and a new flower in the place of carpels, giving rise to indeterminacy of the flower as an additional effect of the absence of *AG* function. Constitutive overexpression of *AG* induces homeotic changes in the flower: carpels instead of sepals and stamens in the place of petals, presenting the characteristics of *apetala2* (*ap2*) mutants (Yanofsky et al., 1990). This *ap2* loss-of-function-like phenotype induced by overexpression of *AG* orthologues in *Arabidopsis* or tobacco is being used as heterologous system for testing functional homology of genes from diverse species such as hazelnut (Rigola et al., 2001), grapevine (Boss et al., 2001), hyacinth (Li et al., 2002) and the conifer black spruce (Rutledge et al., 1998).

Knowledge about MADS-box gene functioning from model species may provide tools for potential applications in important commercial crops. Among them, ornamental species are the most obvious candidates for floral morphology manipulations, in order to create novel varieties with high market values. *Petunia*, which has become another model species for studying MADS-box genes (Angenent et al., 1992, 1995; Ferrario et al., 2003; Kater et al., 1998), *Antirrhinum*, which was used as a model species since the dawn of the ABC model (Coen and Meyerowitz, 1991), rose (Kitahara and Matsumoto, 2000; Kitahara et al., 2001), carnation (Baudinette et al., 2000), gerbera (Yu et al., 1999; Kotilainen et al., 2000), lisianthus (Tzeng et al., 2002), primula (Webster and Gilmartin, 2003), orchids (Lu et al., 1993; Yu and Goh, 2000, 2001; Hsu and Yang, 2002), hyacinth (Li et al, 2002), and lily (Tzeng and Yang, 2001; Tzeng et al., 2002) are among the ornamental crops in which ABCDE model genes were under study.

Lily (*Lilium longiflorum* Thunb.) is one of the most important ornamental species in the world. Tzeng and Yang (2001) and Tzeng et al. (2002) described recently its flower structure and development. Importantly, organs of the two outermost whorls of lily flower are very similar, generating a perianth of tepals, instead of sepals and petals. It has been supposed that this similarity would be due to a modification in the ABC model, leading to an extension of B function towards the first whorl in Liliaceae species (Theissen et al., 2000).

Here we present the results of our investigations on the molecular characterisation of flower development in lily. In order to understand more about floral homeotic genes in this species, we isolated and characterised the MADS-box gene *LLAG1*. Sequence analysis, expression patterns and functional characterisation in the heterologous species *Arabidopsis thaliana* allowed us to conclude that *LLAG1* is the functional *AG* orthologue in lily.

Results

Isolation and sequence analysis of *LLAG1* cDNA from lily.

In order to isolate the *AG* homologue from *Lilium longiflorum,* a cDNA library derived from developing flowers was screened using as probe a 760-bp fragment lacking the MADS-box from *LRAG*, a putative *AG* homologue from *Lilium regale* (Theissen et al., 2000). Five positive clones were selected out of about 200,000 pfu, of which four clones were identical in their sequences and designated as *LLAG1*.

LLAG1 cDNA is 1171 bp long with a 5' leader region of at least 73 bp and a 3' untranslated region of 366 bp upstream the poly(A) tail. Deduced protein sequence analysis of *LLAG1* revealed a 244-amino acid product.

Amino acid sequence alignment of *LLAG1* and *AG* homologues from monocot and dicot species is displayed in Figure 1. A high sequence conservation in the 56 amino acids of the MADS domain is evident, and also to a lesser extent in the K domain, revealing the MIKC structure, typical of type II plant MADS box proteins. An N-terminal extension preceding the MADS domain, commonly present in *AG* homologues, was not found in *LLAG1*. Additionally, an insertion of 21 amino acids was found in the C-terminus of *LLAG1* when compared to *AG,* which is not present in any of the related sequences studied. Additional deletions of about 8 and 4 amino acids in the C-terminus of *LLAG1* in relation to the *AG* sequence could also be found, but these deletions were present in other *AG* paralogues with characterized functions, like *CaMADS1* and *pMADS3*.

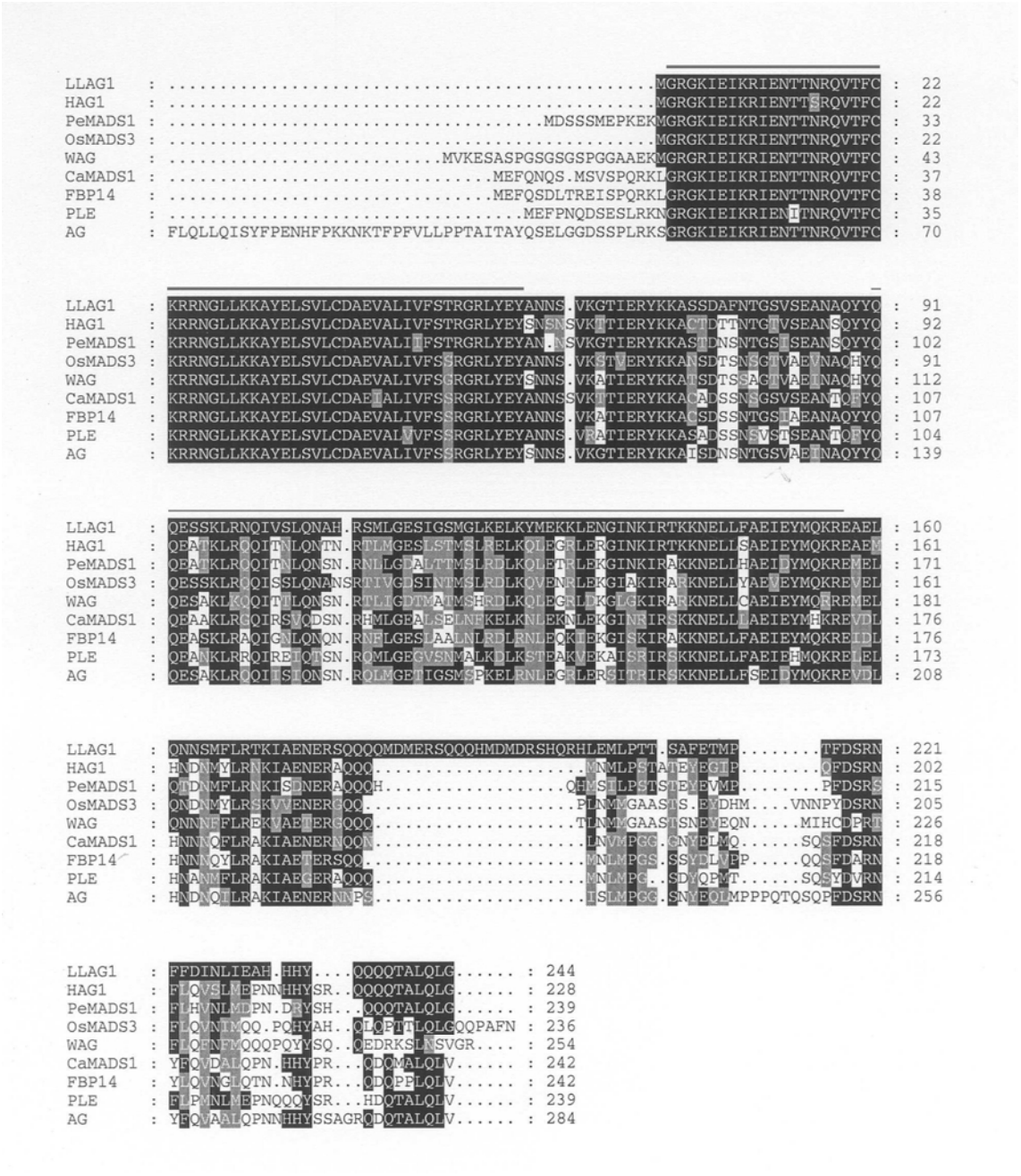
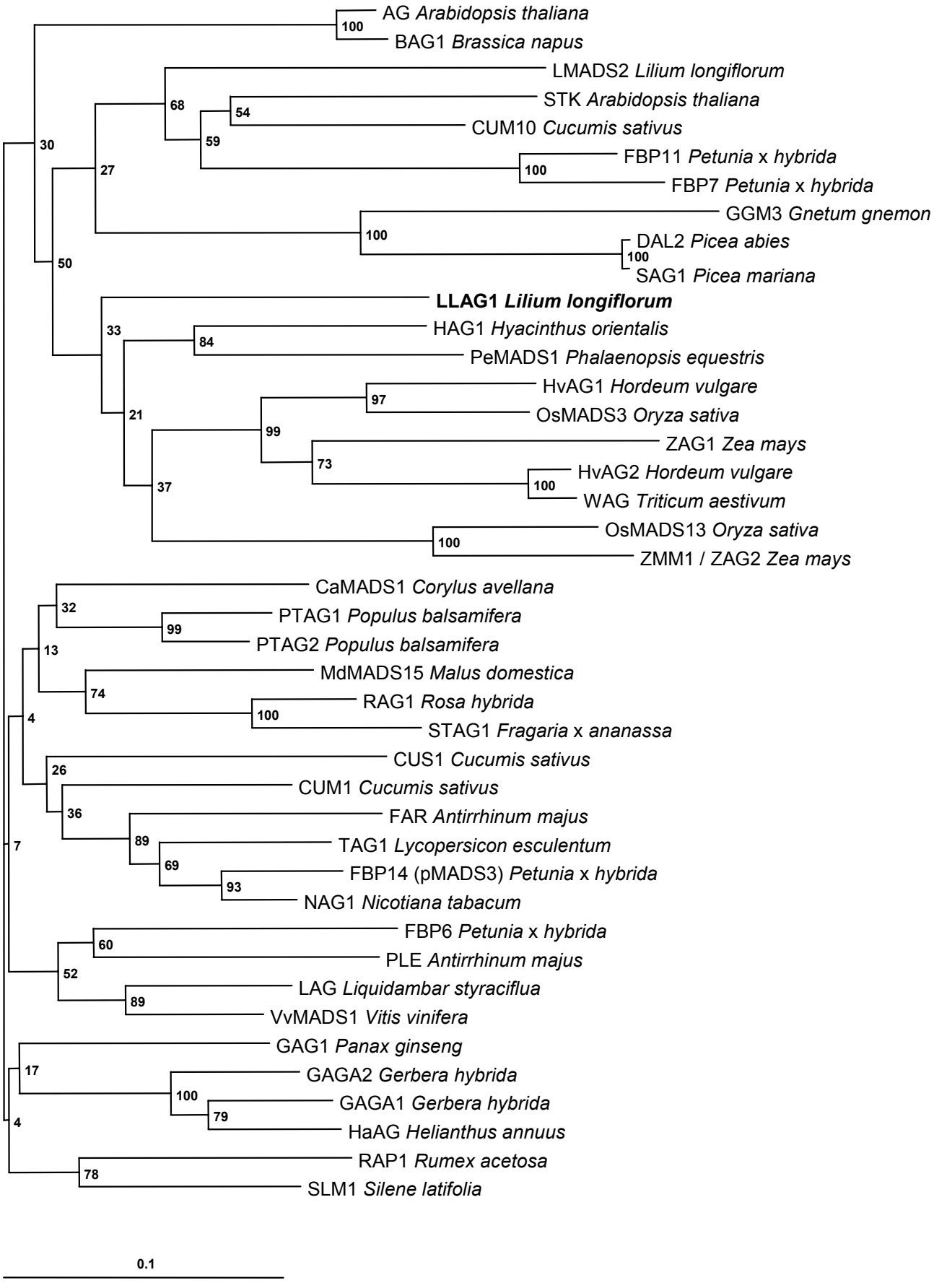


Figure 1. Comparison of deduced amino acid sequences encoded by *LLAG1* and related members of the *AG* subfamily. The alignment was generated by the GCG program and displayed with the GeneDoc program. Identical amino acid residues in relation to *LLAG1* are black and conserved residues are in grey. Dots indicate gaps inserted for alignment optimisation. The amino acid positions are shown on the right. A thick line is drawn above the MADS-box and a thin line above the K-box. Gene codes are described in the Material and Methods section and for species see Figure 2.


AG Arabidopsis thaliana
BAG1 Brassica napus
LMADS2 Lilium longiflorum
STK Arabidopsis thaliana
CUM10 Cucumis sativus
FBP11 Petunia x hybrida
FBP7 Petunia x hybrida
GGM3 Gnetum gnemon
DAL2 Picea abies
SAG1 Picea mariana
LLAG1 Lilium longiflorum
HAG1 Hyacinthus orientalis
PeMADS1 Phalaenopsis equestris
HvAG1 Hordeum vulgare
OsMADS3 Oryza sativa
ZAG1 Zea mays
HvAG2 Hordeum vulgare
WAG Triticum aestivum
OsMADS13 Oryza sativa
ZMM1 / ZAG2 Zea mays
CaMADS1 Corylus avellana
PTAG1 Populus balsamifera
PTAG2 Populus balsamifera
MdMADS15 Malus domestica
RAG1 Rosa hybrida
STAG1 Fragaria x ananassa
CUS1 Cucumis sativus
CUM1 Cucumis sativus
FAR Antirrhinum majus
TAG1 Lycopersicon esculentum
FBP14 (pMADS3) Petunia x hybrida
NAG1 Nicotiana tabacum
FBP6 Petunia x hybrida
PLE Antirrhinum majus
LAG Liquidambar styraciflua
VvMADS1 Vitis vinifera
GAG1 Panax ginseng
GAGA2 Gerbera hybrida
GAGA1 Gerbera hybrida
HaAG Helianthus annuus
RAP1 Rumex acetosa
SLM1 Silene latifolia
100
68
54
59
30
27
100
100
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100
33
84
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97
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4
78
0.1

Figure 2. Phylogram of MADS-box gene members with C and D functions of the *AG* subfamily. The tree was generated by ClustalX version 1.8 program (Thompson et al., 1997) using a Phylip distance matrix with 1,000 bootstrap trials and the graphic representation was given by TREEVIEW software (Page, 1996). *LLAG1* is indicated in bold text. The species of origin are given after the abbreviated name of the genes. Bar represents 10% amino acid substitution per site along the 185-domain.

Within the MADS domain, *LLAG1* shares 100% (56/56) amino acid similarity with *AG* from *Arabidopsis thaliana*, *HAG* from *Hyacinthus orientalis*, *PeMADS1* from *Phalaenopsis equestris*, *OsMADS3* from *Oryza sativa*, *WAG* from *Triticum aestivum*, *pMADS3* from *Petunia hybrida* and *CaMADS1* from *Corylus avellana* while there is only one non-conserved amino acid substitution in *PLE* from *Antirrhinum majus*, leading to 98% similarity. The C-terminus is the least conserved portion of the *AG* homologues. *LLAG1* shares 81% (58/72) amino acid similarity with *HAG1*, 92% (67/73) with *PeMADS1*, 59% (47/80) with *OsMADS3*, 82% (64/78) with *WAG*, 93% (65/70) with *CaMADS1*, 89% (62/70) with *pMADS3*, 84% (59/70) with *PLE*, and 60% (48/80) with *AG*. As a whole, the similarities in the predicted primary structure of *LLAG1* range from 89% with *HAG1* (202/228) to 67% with *AG* (191/284) among the sequences shown in Figure 1.

Phylogenetic analyses indicate that MADS-box sub-families representing monophyletic clades tend to show similar sequences, expression patterns and related functions (Purugganan, 1997). Multiple alignment with *LLAG1* and other members of the monophyletic *AG* clade is presented as a phylogram in Figure 2. It shows that *LLAG1* is closely related to the monocot *AG* orthologues, specially *HAG1* and *PeMADS1*, the latter being from an orchid species.

LLAG1 expression pattern.

The spatial expression pattern of *LLAG1* in floral organs of lily was investigated by RT-PCR using gene specific primers designed to amplify the 3' portion, which is its least conserved section. Amplification of a *GAPDH* fragment was used as a constitutive control. A fragment of approximately 500 bp corresponding to *LLAG1* transcripts could only be detected in stamens and carpels while it was not detectable in tepals or leaves (Figure 3).

This expression pattern suggests that this gene is involved in the development of reproductive floral organs since it is expressed in stamens and carpels but remains

inactive in the perianth and vegetative tissues. These findings are consistent with the hypothesis that *LLAG1* has a similar function as *AG* in lily floral development.

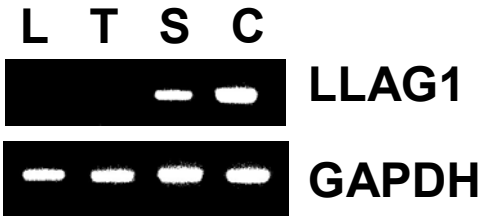

Figure 3. Expression of *LLAG1* in different floral and vegetative organs. RT-PCR using primers for the 3'-terminal portion of *LLAG1* was used for specific amplification. This result indicated that *LLAG1* transcripts are present only in stamen (S) and carpels (C) of the flower and not in leaves (L) or tepals (T). Loading and RNA quality control were verified by amplification of the constitutive lily *GAPDH*.

Ectopic expression of *LLAG1* in *Arabidopsis*.

Functional analysis of *LLAG1* cDNA was done by ectopic expression in *Arabidopsis* to understand whether the sequence and expression similarities between *LLAG1* and *AG* also point to a functional relationship. A binary vector carrying 35S::*LLAG1* and a kanamycin resistance gene was introduced into *Arabidopsis* via *Agrobacterium* transformation and the phenotypic alterations of the transformed plants were analysed in the T_1 and T_2 generations.

According to the ABC model of flower development, transgenic plants overexpressing *AG* are expected to show homeotic modifications in the first and second whorls of the flower. This results in the formation of carpelloid organs in the first whorl and petals replaced by organs with a staminoid identity, acquiring characteristics of the *ap2* mutant phenotype (Bowman et al., 1991), due to the negative interaction between A- and C-functions.

Out of 60 independent kanamycin-resistant plants analysed in the T_1, 26 exhibited phenotypic alterations. Plants showing homeotic changes were divided into strong and weak *ap2*-like phenotypes. In general, strong *ap2*-like plants displayed reduced height, small and curled leaves, loss of inflorescence indeterminacy, bumpy siliques and also flowered earlier than wild type plants (Figure 4), whereas the weak *ap2*-like group showed normal vegetative growth with less pronounced floral homeotic modifications.

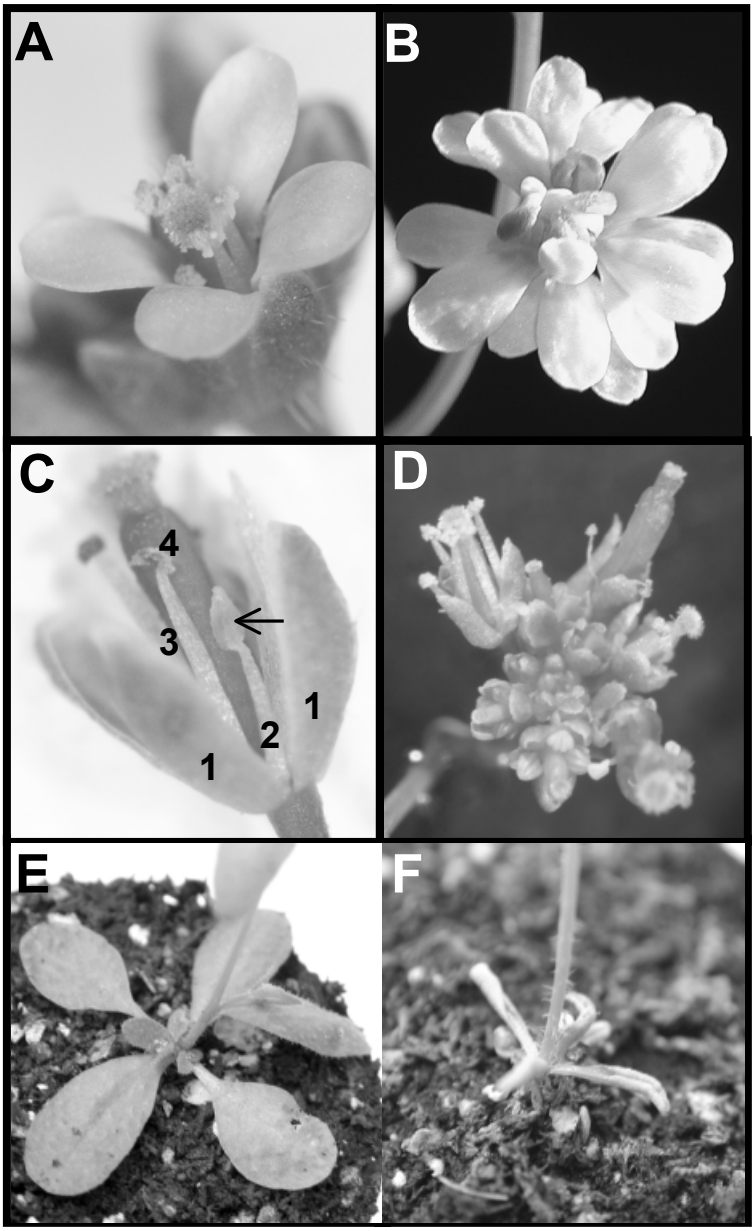

Figure 4. Floral and vegetative morphology of *Arabidopsis*. **A**, Wild-type flower consisting of 4 sepals, 4 petals, 6 stamens and a pistil. **B**, *ag-1* mutant flower in which stamens are converted to petals and the pistil to a new flower in a reiterated manner. **C** and **D**, Transgenic plants overexpressing *LLAG1* under 35S promoter show homeotic mutations in the first and second whorls similar to those found in *ap2* mutants. Arrow indicates a complete conversion of a petal into a stamen in the second whorl, which is visualised in between first whorl organs. Whorl numbers are indicated on the organs. **E**, Rosette leaves of a wild type plant. **F**, Rosette leaves of a transgenic plant with a strong *ap2*-like phenotype.

Flowers derived from plants showing the strong *ap2*-like phenotype had evident homeotic modifications in the first two whorls, with distinct stigmatic papillae at the apex of the modified sepals, and occasionally, complete homeotic changes of petals into stamens. Plants with a weak phenotype often presented partly or completely developed petals.

T_2 progeny analysis was carried out with six self-pollinated strong *ap2*-like T_1 plants. Offspring plants revealed clear segregation of wild type and strong *ap2*-like phenotypes. No weak *ap2*-like phenotype was found in the T_2 progeny analysed. Northern blots confirmed the expression of *LLAG1* in the flowers of T_1 transformed *Arabidopsis* (Figure 5).

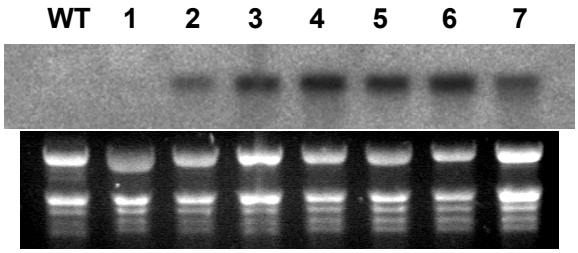

Figure 5. Overexpression of *LLAG1* in *Arabidopsis*. Each lane was loaded with 5 µg total RNA from flowers of the T_1 generation. WT indicates wild type. Plant number 1 showed a normal, wild type phenotype, plants 2 and 3 showed a weak *ap2*-like phenotype while plants 4 to 7 showed strong *ap2*-like phenotypes. Lower panel shows rRNA on agarose gel stained with ethidium bromide for loading control.

Discussion

Since lily (*Lilium* sp.) is one of the major ornamental crops in the world and ABCDE model genes are involved in the morphology of the flower, research on those genes can be of great commercial interest. Evolutionary and developmental biology may also take advantages from these studies since not many floral homeotic genes from monocot species have been investigated in detail so far.

LLAG1, a *Lilium longiflorum* MADS-box gene isolated from a cDNA library from developing flowers, is specifically expressed in the stamens and carpels, constituting the C domain of the ABCDE model for floral development.

The primary structure of *LLAG1* protein showed to be highly homologous to *AGAMOUS* from *Arabidopsis* and other known orthologues. As expected, the phylogenetic dendrogram revealed a close relationship to *AG* orthologues from monocot species.

Although no specific function was given so far to the N-terminal extension of the MADS domain, since *AG* with truncated N-terminus still showed to be functionally active *in vitro* (Pollock and Treisman, 1991; Huang et al., 1993), it was suggested that all functional *AG* homologues would contain this extension (Kater et al., 1998). However, it was found that the *AG* homologues *HAG1* from hyacinth (*Hyacinthus orientalis*), *OsMADS3* from rice (*Oryza sativa*) and *CUM10* from cucumber (*Cucumis sativus*) have their presumed start codon at the MADS-box. In addition, besides showing a putative N-terminal portion before the MADS domain, *PeMADS1* from *Phalaenopsis equestris* and *WAG* from wheat (*Triticum aestivum*) contain an ATG codon just in front of their MADS-box, which could be their actual start codon. *LLAG1* does not carry an N-terminal domain preceding the MADS domain, indicating that this extension might have been abolished during plant evolution, possibly due to its lack of functionality. Furthermore, an insertion of 21 amino acids was found in the C-terminal portion of *LLAG1* that is not present in the other orthologues analysed.

Based on RT-PCR, the spatial expression of *LLAG1* in mature flowers corresponded to the *AG* expression in *Arabidopsis* (Yanofsky et al., 1990), being expressed in the third and fourth whorl of the flower in accordance to the ABC model.

Due to the very low transformation efficiency of lily, functional studies of *LLAG1* were undertaken in the model species *Arabidopsis*. Even with the insertion of 21 amino acids in its C-terminus, constitutive overexpression of *LLAG1* led to homeotic changes of floral organs in *Arabidopsis*. The modifications observed were entirely in accordance to reports on *AG* overexpression in *Arabidopsis* (Mizukami and Ma, 1992) and functional analyses of *AG* orthologues from hazelnut (Rigola et al., 2001), hyacinth (Li et al., 2002) and spruce (*Picea mariana*; Rutledge et al., 1998).

The complete homeotic changes of petals into stamens in *Arabidopsis* flowers due to ectopic expression of *LLAG1* provided additional indications of the capability for *in vivo* cross-interaction of proteins belonging to the ABC model from different species, even with those distantly related, like *Arabidopsis* and lily. It can also reiterate the evolutionary importance of *AG* function in flowering plants due to the preservation of *in vivo* functionality of protein-protein interaction among MADS-box transcription factors from diverse species. Molecular evidences of cross-interactions among several MADS-box proteins from *Arabidopsis* and Petunia (Immink and Angenent, 2002) and also between Petunia and rice (Favaro et al., 2002) were provided in recent studies. However, there are suggestions that MADS-box protein dimerization may occur in a different way in monocot species (Winter et al., 2002).

There are several sequences from ABCDE class of genes from bulbous crops, such as the *AG* orthologue *HAG1* from hyacinth (Li et al. 2002), the B-type genes

LMADS1 from *L. longiflorum* (Tzeng and Yang, 2001) and *LRDEF*, *LRGLOA* and *LRGLOB* from *L. regale* (Winter et al., 2002), and a partial sequence of *PeMADS1* from an orchid (*Phalaenopsis equestris*) available in the public gene database. Recently, *LMADS2*, a new MADS-box gene from *L. longiflorum* was described as a potential class D gene (Tzeng et al., 2002). However, functional analysis of *LMADS2* in *Arabidopsis* induced the same characteristics found when overexpressing the C type *AG* or its orthologues instead of promoting ectopic ovule formation as it was observed with other D functional genes, such as *FBP11* from Petunia (Colombo et al., 1995).

Among the oriental hybrids of *Lilium* sp. currently in the market, there are double flower varieties that resemble the *AG* loss of function, having tepals instead of stamens and a new flower instead of carpels with loss of flower determinacy. This variant pattern arises naturally and occurs for many species, being of great interest of flower breeders for creating novel characteristics. Nevertheless, the phenomenon is very rare and not completely elucidated yet. It can be due to mutations in the *AG* orthologue directly or even caused by abnormal interactions with trans-regulatory elements (Roeder and Yanofsky, 2001; Franks et al., 2002). Knowledge about the mechanisms involved in this process would allow modifying crops, such as lily, to create new varieties with a double flower phenotype.

Material and Methods

Plant material. Lily (*Lilium longiflorum* Thunb. cv. Snow Queen) plants used in this study were grown in greenhouse conditions (18-25^{o}C day/14-18^{o}C night with natural light regime during growth season) in Wageningen, the Netherlands. *Arabidopsis thaliana*, Columbia (Col) ecotype were grown in the greenhouse under long day regime (22^{o}C, 14 h light/10 h dark) after breaking the dormancy of the seeds by 3 days at 4^{o}C.

RNA extraction. Total RNA was extracted from lily floral buds (1.0 to 3.5 cm), leaves and mature floral organs (tepal, stamen and pistil) according to Zhou et al. (1999). *Arabidopsis* total RNA from flowers was isolated using the Rneasy Plant Mini kit (Qiagen, GmbH, Hilden, Germany).

cDNA library preparation and screening. cDNA was synthesised using the ZAP-cDNA Gigapack III Gold Cloning Kit (Stratagene, La Jolla, CA) with 5 μg of a poly(A)$^{+}$ RNA pool from 1.0 to 3.5 cm lily buds purified through the Poly(A) Quik mRNA

isolation column (Stratagene). cDNA fractions containing 1 to 1.5 kb fragments were unidirectionally inserted between the *EcoRI* and *XhoI* sites of the Uni-ZAP XR phage vector.

Approximately 200,000 pfu were screened using a 760 bp fragment of *LRAG*, a putative *AG* homologue from *Lilium regale* (Theissen et al., 2000), without the MADS-box, labelled with [^{32}P] dATP using RadPrime DNA Labeling System (Invitrogen, Carlsbad, CA). Hybridisation procedures were carried on at 56^{o}C overnight on nylon membranes and washes reached the stringency of 0.5x SSC, 0.1% SDS. Blots were exposed to X-ray films for 24 h and positive clones were collected from plates for *in vivo* excision procedures.

Sequence analysis. cDNA clone sequencing was performed in both directions with T3 (5'-AAT TAA CCC TCA CTA AAG GG-3') and T7 (5'-GCC CTA TAG TGA GTC GTA TTA C-3') primers using rhodamine dye (Applied Biosystems, Foster City, CA). Sequence analyses were carried out with DNASTAR software package.

Multiple protein sequence alignment was performed using the 185-domain (185 amino acids starting from the MADS-box) (Rigola et al., 1998). The accession numbers of the protein sequences used in this study are as followed: *AG* (CAA37642); *AGL11* (AAC49080); *BAG1* (AAA32985); *CaMADS1* (AAD03486); *CUM1* (AAC08528); *CUM10* (AAC08529); *CUS1* (CAA66388); *DAL2* (CAA55867); *FAR* (CAB42988); *FBP6* (CAA48635); *FBP7* (CAA57311); *FBP11* (CAA57445); *FBP14* (*pMADS3*; CAA51417); *GAG1* (CAA86585); *GAGA1* (CAA08800); *GAGA2* (CAA08801); *GGM3* (CAB44449); *HAG1* (AAD19360); *HaAG* (AAN47198); *HvAG1* (AAL93196); *HvAG2* (AAL93197); *LAG* (AAD38119); *LMADS2* (Tzeng et al., 2002); *MdMADS15* (CAC80858); *NAG1* (AAA17033); *OsMADS3* (AAA99964); *OsMADS13* (AAF13594); *PeMADS1* (AAL76415); *PLE* (AAB25101); *PTAG1* (AAC06237); *PTAG2* (AAC06238); *RAG1* (AAD00025); *RAP1* (CAA61480); *SAG1* (AAC97157); *SLM1* (CAA56655); *STAG1* (AAD45814); *TAG1* (AAA34197); *VvMADS1* (AAK58564); *WAG* (BAC22939); *ZAG1* (AAA02933) and *ZMM1* (considered here equivalent to *ZAG2*, CAA56504). The sequence of the putative *AG* homologue from *Lilium regale* (*LRAG*) (Theissen et al., 2000) was not included in the alignment because 185 domain sequence data for the entry was not made publicly available.

Expression analysis. RT-PCR analysis was performed using SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen). Five micrograms of total RNA extract from floral organs and leaves were used to synthesise the first-strand cDNA using an

oligo-dT primer. For amplification of *LLAG1* cDNA, a 2 µL aliquot of the first-strand RT reaction were used in a 50 µL PCR reaction with *LLAG1* specific primers (5'-GAT TGC TGA AAA TGA GAG G-3' and 5'-AAA GTC ACA AAA TAA TAC AGC-3' as forward and reverse primer, respectively). PCR was performed with 10 min 95°C denaturation step, followed by 35 cycles of 1 min at 95°C, 1 min annealing at 54°C and 1 min extension at 72°C and a final extension period of 10 min. Twelve microliters of the RT-PCR reaction were run in an agarose gel and photographed under UV light. As a control, 2 µL of the first-strand RT reaction was used for amplification of the constitutive glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) cDNA using degenerated primers (5'-GTK GAR TCN ACY GGY GTC TTC ACT-3' and 5'-GTR TGR AGT TGM CAN GAR ACA TC-3' as forward and reverse primers, respectively) conceived from alignment of *GAPDH* sequences of several monocot species available in the GenBank. The PCR conditions for *GAPDH* were as described for *LLAG1*.

Northern blot of transgenic *Arabidopsis* was prepared with 5 µg total RNA from flowers of first generation transformants. Probes were prepared as described for cDNA screening, using *LLAG1* without the MADS-box and, as constitutive expression control, a 1.2 kb actin cDNA fragment, obtained by RT-PCR amplification of leaf RNA with specific primers (5'-GCG GTT TTC CCC AGT GTT GTT G-3' and 5'-TGC CTG GAC CTG CTT CAT CAT ACT-3' as forward and reverse primers, respectively). Hybridisation was carried out at 60°C and washes reached 0.5 x SSC, 0.1% SDS at the same hybridisation temperature in both cases. After *LLAG1* hybridisation and development, the membrane was stripped with 0.1 x SSC, 0.1% SDS at boiling temperature for 20 min. The membrane was exposed for 4 h to Phosphor Imaging Plate (Fuji Photo Film Co., Tokyo, Japan) and developed in a related scanner system. Autoradiographic signals were processed using the TINA 2.10 software (Raytest, Straubenhardt, Germany).

Binary vector construction and *Arabidopsis* transformation. A pBINPLUS-derived binary vector (van Engelen et al., 1995) with a multiple cloning site between the CaMV 35S promoter and the *NOS* terminator was used for sense-oriented *LLAG1* insertion and overexpression in *Arabidopsis*. *LLAG1* was excised from the pBluescript SK^+ vector using *XbaI* and *XhoI* restriction enzymes and inserted into the binary vector treated with the same enzymes. After confirmation of the sequence, *Agrobacterium tumefaciens* strain C58C1 competent cells were prepared and transformed by electroporation according to Mattanovich et al. (1989).

Arabidopsis thaliana ecotype Columbia (Col) plants were transformed using the floral dip method (Clough and Bent, 1998). T_1 seeds were placed onto agar plates

containing 0.5X MS medium (Murashige and Skoog, 1962) with 50 μg mL^{-1} kanamycin as selection agent. In order to break the dormancy, plates were placed at 4 ^{o}C for 3 days and then at 23 ^{o}C in a growth chamber with long-day conditions (14 h light/ 10 h dark). T_1 seedlings were transferred to soil and kept in the same temperature and light regime. Self-pollinated T_2 population was sown directly in soil and grew in identical conditions.

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CHAPTER THREE

Overexpression of a MADS-box gene from *Lilium longiflorum* homologous to *SEPALLATA3* is able to induce early flowering in *Arabidopsis*

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Abstract

SEPALLATA3 (*SEP3*) is a MADS-box homeotic gene possibly determining the E function in the ABCDE model. This function is essential for proper development of petals, stamens and carpels. In order to gain further information on lily (*Lilium longiflorum*) flower development at the molecular level, a cDNA library constructed from developing floral buds was screened, and a clone (*LLSEP3*) was isolated with high similarity to the *SEP3* transcription factor from *Arabidopsis*. *LLSEP3* belongs to the *AGL2* subfamily of MADS-box genes and shares its closest relationships with *DOMADS1* and *OM1*, from the orchid species *Dendrobium grex* and *Aranda deborah*, respectively. Expression analysis by Northern hybridisation showed that *LLSEP3* was expressed throughout lily flower development and in tepals, stamens and carpel tissues of mature flowers, whereas no expression was detected in leaves. Overexpression of *LLSEP3* in *Arabidopsis* under the CaMV35S promoter induced early flowering but did not induce any floral homeotic changes, which is in accordance with the effect of *SEP3* overexpression in this species. Altogether, these data are consistent with the putative role of *LLSEP3* as an E functional gene in lily flower development.

Introduction

Transcription factors play important roles in many biological processes, binding to regulatory regions of the DNA and to other protein factors in order to inhibit or assist RNA polymerase in initiation or maintenance of transcription. The MADS-box genes represent a large family of highly conserved transcription factors found in plants, animals and yeast involved in a range of developmental processes (Riechmann and Meyerowitz, 1997; Schwarz-Sommer et al., 1990; Theissen et al., 2000). In plants, they play pivotal roles in the regulation of flowering time, meristem identity, floral organ and fruit development (Causier et al., 2002) and, next to these reproductive functions, they are also active in root architecture (Zhang and Forde, 1998) and may be necessary for guard cell and trichome development, as deduced by expression analysis (Alvarez-Buylla et al., 2000).

Flower development has fascinated biologists for a long time but only in the last decade a neat, elegant and satisfactory model for it was conceived. The identified genes from the ABC model work in a combinatorial way for proper floral organ development in which A type genes lead to sepal formation, A and B type genes together trigger petal development, B and C type form stamens and the C type gene expression constitutes carpels (Coen and Meyerowitz, 1991). The studies were first performed in *Arabidopsis thaliana* and *Antirrhinum majus*, but soon they were extrapolated to many other plants, confirming that the ABC model was a general model for flower development throughout angiosperm species. However, the simplicity of the original model demanded further elaboration to cope with the complexity involved in flower development. This led to the addition of new functions to the model, like the D function, which is involved in ovule development (Angenent et al., 1995), and the E function, which was shown to be essential for petal, stamen and carpel formation (Jack, 2001; Pelaz et al., 2000). The ABCDE model is of great value for developmental studies and many MADS-box orthologues have been studied in other species, including monocots such as sugarcane, sorghum, maize and rice (Dornelas and Rodriguez, 2001; Greco et al., 1997; Mena et al., 1995; Pelucchi et al., 2002).

One hundred and seven MADS-box genes have been identified in the *Arabidopsis* genome (Parenicová et al., 2003). The MADS domain comprehends 56 amino acids and is the most conserved portion of the protein, which is involved in DNA binding at the cis-elements motifs known as CArG boxes (Schwarz-Sommer et al., 1990; Treisman, 1990). Many MADS-box genes contain a so-called MIKC structure, presenting additionally to the MADS-box, a K-box that is a conserved domain capable of mediating protein-protein interactions. The carboxy-terminal

portion is the least conserved and is involved in transcription activation and specificity of the protein function (Riechmann and Meyerowitz, 1997).

Three MADS-box genes are responsible for the E function in *Arabidopsis*, the *SEPALLATA* (*SEP*) *1*, *2* and *3* (previously referred to as *AGL2*, *4* and *9*), playing redundant roles throughout floral organ formation. *SEP1* and *2* are expressed in every whorl of the flower (Flanagan and Ma, 1994; Savidge et al., 1995) while *SEP3* is expressed in the petals, stamens and carpels (Mandel and Yanofsky, 1998). Homeotic triple *sep1/2/3* mutant led to the formation of sepaloid structures in all whorls and the loss of floral determinacy, resembling double B and C loss-of-function phenotype (Pelaz et al., 2000), while the overexpression of *SEP3* in sense orientation did not lead to any homeotic mutation, but to an early flowering phenotype. *SEP3* overexpression in antisense orientation led to the resemblance of *AP1* intermediate alleles (Pelaz et al., 2001a), suggesting an additional role of *SEP3* in floral transition (Mandel and Yanofsky, 1998). The most striking observation was, however, that overexpression of *SEP* genes concomitantly with the overexpression of A and B type genes resulted in the conversion of leaves into petals, revealing the *SEP* function as the missing factor to trigger this metamorphosis (Honma and Goto, 2001; Pelaz et al., 2001b).

Besides the E function of the ABCDE model of flower development is already well established in model species, such as *Arabidopsis* and petunia, this function is not much known in other species yet, and this is particularly true for monocots. In rice, a model species for the ABCDE model in monocots, some genes were identified as related to *SEP* genes, based on their sequences and expression pattern, such as *OsMADS1* (*LEAFY HULL STERILE1, LHS1*) (Jeon et al., 2000), *OsMADS24* (also known as *OsMADS8*), *OsMADS34* and *OsMADS45* (also referred as *OsMADS7*) (Fornara et al., 2003; Kang and An, 1997; Kang et al., 1997).

The plant MADS-box genes were classified into several groups according to their amino acid sequence, indicating their evolutionary relationships. The *AP1*/*AGL9* family (Purugganan et al., 1995) contains the majority of MADS-box genes involved in floral transition and the A and the E functional genes. It can be divided into the *AGL2*, *SQUA* (Theissen et al., 1996, 2000) and *OsMADS1* (Yu and Goh, 2000) subfamilies.

Despite their sequence similarities and evolutionary relationships, functional resemblance can only be tested by *in vivo* methods. Studies involving petunia MADS-box genes and yeast two-, three and four-hybrid analyses indicated that protein interaction in yeast is a good system for functional homology characterisation of plant MADS-box proteins (Favaro et al., 2002; Ferrario et al., 2003, Immink and Angenent, 2002; Immink et al., 2003).

The ABCDE model is particularly interesting to be investigated in members of the Liliales order, such as lilies, tulips and orchids. It has been proposed that they would show an altered expression pattern of the genes involved in petal formation (van Tunen et al., 1993, referring to tulips) because their flowers show petaloid organs throughout the entire perianth. Moreover, characterisation of MADS-box genes involved in flower development may also be useful for practical purposes, such as manipulation of floral structure or flowering time, since many of these species are important ornamental species. In addition, fundamental studies on species divergence and its developmental implications (evolutionary developmental genetics, Theissen et al., 2000) could use the data generated by molecular and functional characterisation of MADS-box genes from member of the Liliales order (Yu and Goh, 2001).

Some MADS-box genes from lily have already been isolated and, to some extent, functionally characterized. Several MADS-box genes from *Lilium regale* were isolated, sequenced and analysed by phylogenetic comparison (Theissen et al., 2000). *LRGLOA* and *LRGLOB*, two *PISTILLATA* (*PI*) putative orthologues from *L. regale*, were shown to form homodimers *in vitro*, what is significant since the corresponding orthologues from dicot species are only able to form heterodimers with B functional proteins. On the other hand, *LRDEF*, a putative *AP3* orthologue, like its counterparts in model species, is only able to form heterodimers in *in vitro* conditions (Winter et al., 2002). These results indicate that the ABCDE model may work differently in lily species and that this mechanistic alteration could have led to the transition of the first whorl organs into petal-like structures. *LMADS1*, from *Lilium longiflorum*, was functionally characterized as a B functional gene and is homologous to *AP3* from *Arabidopsis* (Tzeng and Yang, 2001). *LLAG1,* an orthologue of *AGAMOUS* (*AG*), the C functional gene from *Arabidopsis*, is actively transcribed in developing lily buds, and is able to induce homeotic changes of petals into stamens and of sepals into carpelloid structures, when ectopically expressed in *Arabidopsis*, indicating functional homology to *AG* (this thesis). *LMADS2*, that shared high similarity with the D functional genes *AGL11* from *Arabidopsis* and with *FBP7* and *11* from petunia, mimicked the C function when ectopically expressed in *Arabidopsis* (Tzeng et al., 2002). However, the E function was not functionally characterized earlier in any of the Liliales species and it is interesting to find whether the E genes fulfil a similar role.

Given the significance of lily (*Lilium longiflorum)* as an ornamental crop, having its flower development unravelled at a molecular level would be of great value, which could make available tools for certain applications such as flowering time and floral morphology manipulations. Here, we describe the molecular characterisation and overexpression of a putative *SEP3* homologue from lily, *LLSEP3*. Altogether, its

sequence analyses, transcription profile and the phenotype generated by its overexpression in *Arabidopsis* indicate that *LLSEP3* is the functional orthologue of *SEP3* in lily.

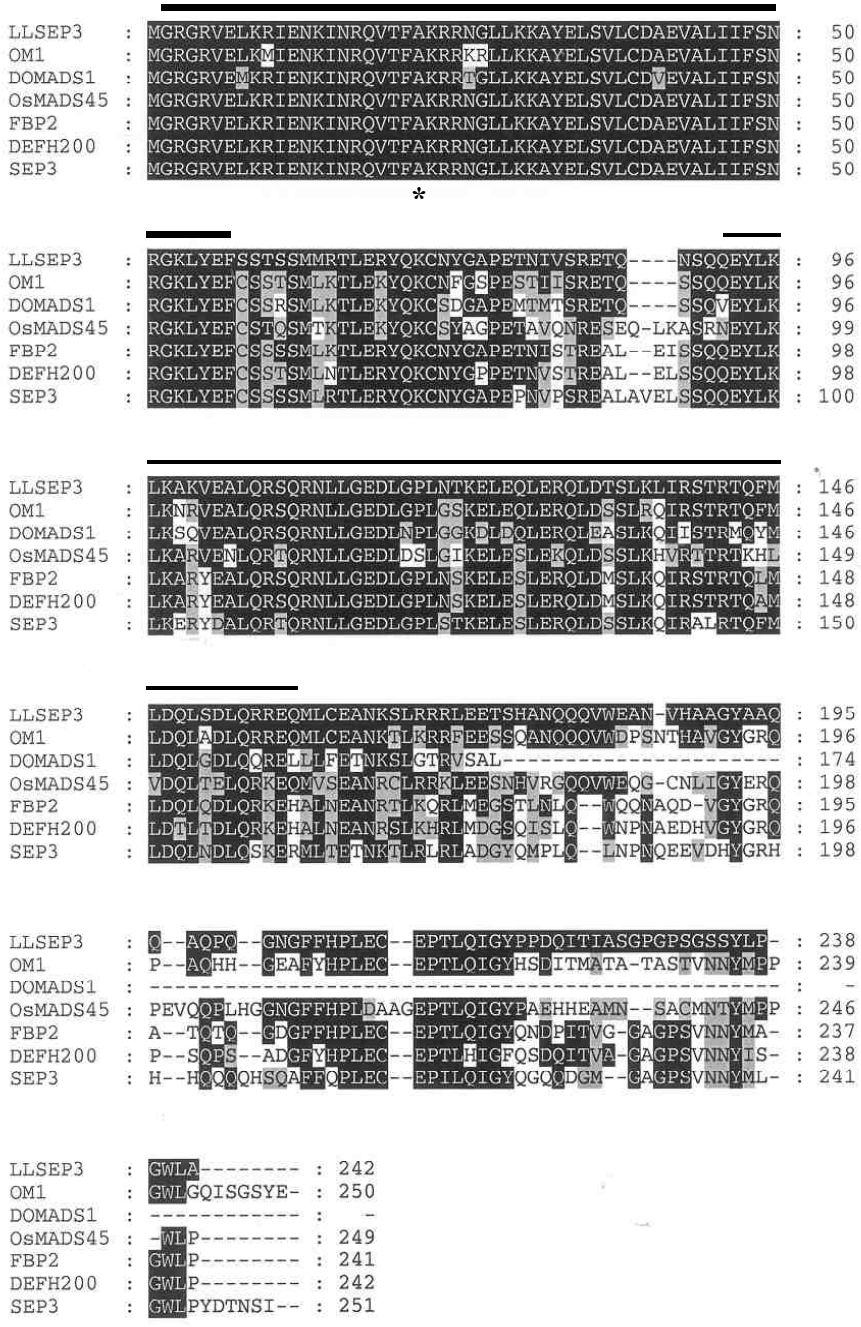

Figure 1. Alignment of deduced amino acid sequences encoded by *LLSEP3* and genes belonging to the *AGL2* MADS-box subfamily. The alignment was generated by the GCG program and displayed with the GeneDoc program. Amino acid identity and conservation in relation to *LLSEP3* is displayed in black and grey, respectively. A thick line is drawn above the MADS domain and a thin line above the K domain. An asterisk is under the conserved alanine residue that defines the *AGL2* subfamily. Gaps are indicated by dashes and were introduced to maximise alignments. Genes are related to species in Figure 2, whereas the GenBank entries are described in the Material and Methods section.

Results and Discussion

Isolation and sequence analysis of the *SEP3* homologue from *Lilium longiflorum*

In order to identify an orthologue of *SEP3* in lily, a cDNA library was screened using a lily probe amplified by RT-PCR with degenerated primers. To avoid cross-hybridisation with other MADS-box genes, these primers were conceived from sequence alignment of the carboxy-terminal portion from several putative monocot *SEP3* homologues. Seven positive clones were isolated from about 50,000 pfu screened under stringent conditions. Sequencing showed that all the clones were identical except for the length of their 3' untranslated ends. The longest clone was taken for further studies.

The clone, designated *LLSEP3*, is 1,116 bp long, including a 5' leader region and a untranslated trailer region at the 3' end with the poly(A) tail. Conceptual translation revealed a protein of 242 amino acid residues and a mass of 27.6 kD.

LLSEP3 amino acid sequence and an alignment with related sequences are shown in Figure 1. Analysis of the *LLSEP3* sequence organisation revealed the MIKC structure, as seen in type II MADS-box genes. The 56 residues of the MADS-box domain formed the most conserved region found in *LLSEP3*, presenting 100% (56/56) amino acid similarity with *DOMADS1*, *OsMADS45*, *FBP2*, *DEFH200* and *SEP3*, from *Dendrobium grex*, *Oryza sativa*, *Petunia hybrida*, *Antirrhinum majus*, and *Arabidopsis thaliana*, respectively. The K-box followed as the second most conserved domain in *LLSEP3*, showing amino acid similarity ranging from 74% (42/57) with *OsMADS45* to 95% (53/56) with *OM1* from *Aranda deborah*. The carboxy-terminus was a weakly conserved portion of MADS-box genes; *LLSEP3* shared amino acid identities ranging from 50% (60/120) with *SEP3* to 64% (30/47) with *DOMADS1* whereas the amino acid similarities are between 65% (78/120) with *SEP3* and 78% (94/121) with *OsMADS45*. Taken over the entire protein length, *LLSEP3* shared the highest identity score with *DOMADS1* and *FBP2*, both showing identities of 76% (133/174 and 183/241, respectively) in their sequences, while the highest and lowest amino acid similarities were shared with *DOMADS1* (88%, 153/174)) and *SEP3* (78%, 195/251), respectively. One could wonder whether *DOMADS1* is able to perform properly as an E functional gene with the deletion shown in its carboxy-terminus. The answer is still elusive, since there are not many *SEP3* homologues functionally characterized so far and *DOMADS1* was not functionally analysed yet. *LLSEP3* shows a carboxy terminus corresponding in size to the functionally characterized *FBP2* (Angenent et al., 1995).

Phylogenetic analysis based on the first 185 amino acid residues from the MADS domain revealed that *LLSEP3* belongs to the *AGL2* subfamily of MADS-box

genes, the same clade that encloses the E functional genes such as *SEP3*, *FBP2*, *TM5* (Figure 2). *LLSEP3* shared the closest relationship with *OM1* and *DOMADS1*, from the orchid species *Aranda deborah* and *Dendrobium grex*, respectively.

Temporal and spatial expression of *LLSEP3*

To identify the expression profile of *LLSEP3*, northern blots of total RNA from developing floral buds and mature tissues from lily were hybridised with the 497-bp fragment between the carboxy-terminus and the poly(A) tail of *LLSEP3*.

Floral tissues of developing buds ranging from 1 to 3.5 cm showed strong expression of *LLSEP3* as well as tepals, stamens and carpels of mature flowers (Figure 3). Leaves did not show detectable expression of this gene. *LLSEP3* transcripts are present throughout flower development in all whorls of a mature flower, with a particular higher expression in the tepals.

Expression seemed higher in tepals than in the sexual organs. An expression pattern which is present during initiation of flower development and remains present in mature floral organs is also found typically for type E orthologues, such as *FBP2, SEP3* and *TM5* (Angenent et al., 1992; Mandel and Yanofsky, 1998; Pnueli et al., 1994).

Ectopic expression of *LLSEP3* in *Arabidopsis*

Given that gene function cannot be predicted solely by its sequence and expression pattern, *LLSEP3* overexpression in the heterologous species *Arabidopsis* was followed.

In vivo functional characterisation of *LLSEP3* was carried out in the heterologous species *Arabidopsis*. Twenty five transgenic plants were analysed in the T_1 generation and followed up to the second generation. *LLSEP3* overexpression in the sense orientation under the constitutive CaMV35S promoter led to plants with very early flowering and curled leaves in fifteen of the plants analysed. A representative early flowering plant is shown in Figure 4. Flowers showed the normal phenotype, carrying all four organs. This is consistent with reports of overexpressing *SEP3* and its orthologues in *Arabidopsis* (Ferrario et al., 2003; Pelaz et al., 2001a).

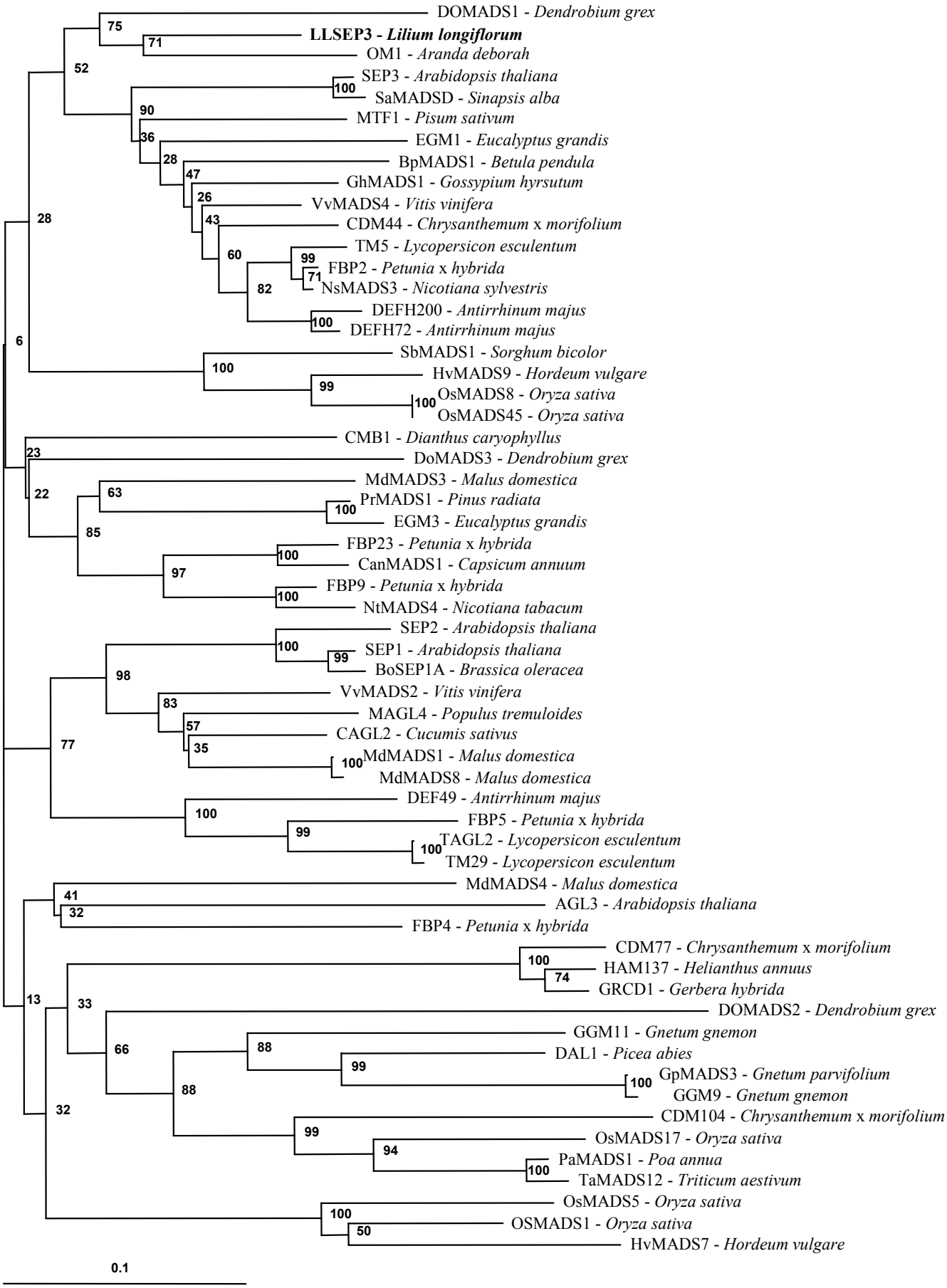
DOMADS1 - Dendrobium grex
LLSEP3 - Lilium longiflorum
OM1 - Aranda deborah
SEP3 - Arabidopsis thaliana
SaMADSD - Sinapsis alba
MTF1 - Pisum sativum
EGM1 - Eucalyptus grandis
BpMADS1 - Betula pendula
GhMADS1 - Gossypium hyrsutum
VvMADS4 - Vitis vinifera
CDM44 - Chrysanthemum x morifolium
TM5 - Lycopersicon esculentum
FBP2 - Petunia x hybrida
NsMADS3 - Nicotiana sylvestris
DEFH200 - Antirrhinum majus
DEFH72 - Antirrhinum majus
SbMADS1 - Sorghum bicolor
HvMADS9 - Hordeum vulgare
OsMADS8 - Oryza sativa
OsMADS45 - Oryza sativa
CMB1 - Dianthus caryophyllus
DoMADS3 - Dendrobium grex
MdMADS3 - Malus domestica
PrMADS1 - Pinus radiata
EGM3 - Eucalyptus grandis
FBP23 - Petunia x hybrida
CanMADS1 - Capsicum annuum
FBP9 - Petunia x hybrida
NtMADS4 - Nicotiana tabacum
SEP2 - Arabidopsis thaliana
SEP1 - Arabidopsis thaliana
BoSEP1A - Brassica oleracea
VvMADS2 - Vitis vinifera
MAGL4 - Populus tremuloides
CAGL2 - Cucumis sativus
MdMADS1 - Malus domestica
MdMADS8 - Malus domestica
DEF49 - Antirrhinum majus
FBP5 - Petunia x hybrida
TAGL2 - Lycopersicon esculentum
TM29 - Lycopersicon esculentum
MdMADS4 - Malus domestica
AGL3 - Arabidopsis thaliana
FBP4 - Petunia x hybrida
CDM77 - Chrysanthemum x morifolium
HAM137 - Helianthus annuus
GRCD1 - Gerbera hybrida
DOMADS2 - Dendrobium grex
GGM11 - Gnetum gnemon
DAL1 - Picea abies
GpMADS3 - Gnetum parvifolium
GGM9 - Gnetum gnemon
CDM104 - Chrysanthemum x morifolium
OsMADS17 - Oryza sativa
PaMADS1 - Poa annua
TaMADS12 - Triticum aestivum
OsMADS5 - Oryza sativa
OSMADS1 - Oryza sativa
HvMADS7 - Hordeum vulgare
0.1

Figure 2. Phylogenetic analysis of MADS-box genes. The tree was generated by the ClustalX version 1.8 program (Thompson et al., 1997) using a Phylip distance matrix with 1,000 bootstrap trials. Graphic representation was produced by the TreeView software (Page, 1996). *LLSEP3* is indicated in bold. The species of origin are given after the respective gene names. Bars represent 10% amino acid substitution per site along the 185 domain.

An explanation of why *SEP3* induces early flower phenotype cannot be easily given up till now. This phenomenon may be due to its intrinsic and uncharacterised role during floral meristem identity or even with functional mimicry of a floral meristem gene, such as *AP1*. Segregation of early flowering and twisted leaves was followed up and confirmed until the T_2 generation. T_1 generation plants were assessed for RNA expression, showing active transcription of *LLSEP3* and the selection *NPTII* gene (Figure 5).

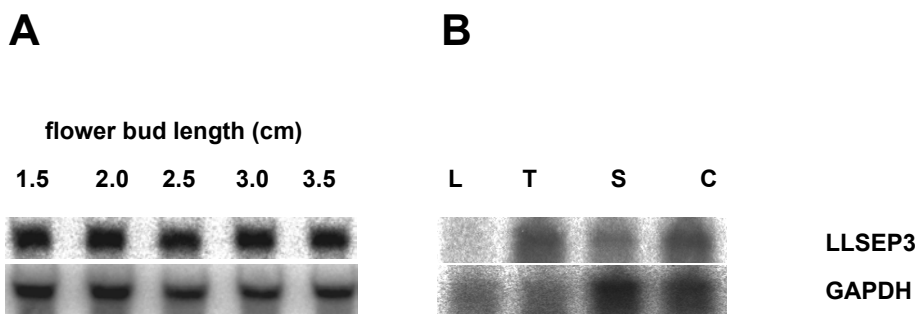

Figure 3. Expression profile analysis of *LLSEP3*. **A.** *LLSEP3* transcripts are present in developing floral buds (1.5 to 3.5 cm lengths). **B.** *LLSEP3* is expressed in mature floral organs (T, tepals; S, stamens; C, carpels) and not detected in leaves (L). Ten micrograms of total RNA were loaded in each lane. Loading control is given by hybridisation with a lily *GAPDH* probe in the lower panels.

Whereas antisense expression of *SEP3* in *Arabidopsis* led to phenotypes similar to intermediate alleles of the flower meristem identity and homeotic gene *ap1* (Pelaz et al., 2001a), antisense expression of *LLSEP3* in *Arabidopsis* did not show any visible phenotypic alteration, presumably because its sequence already diverges too much to promote gene silencing of *SEP3* by antisense RNA.

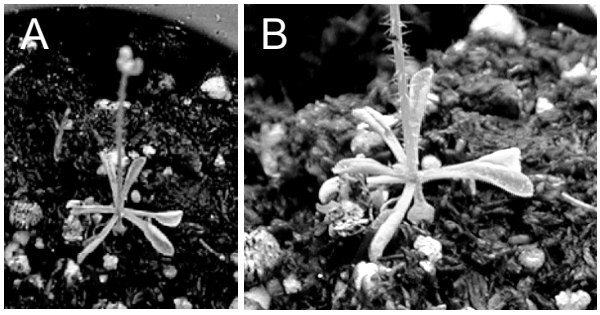

Figure 4. Phenotype of a T_1 *Arabidopsis* overexpressing *LLSEP3*. **A.** Plants were very weak, showing very early flowering and curled leaves. **B.** Close-up of plant shown in picture A. Among 25 transgenic plants analysed, 15 plants showed this phenotype and were followed up to the next generation, which presented this phenotype in most of the plants.

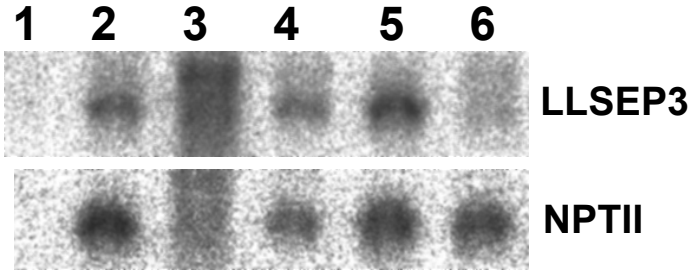

Figure 5. Northern blot analysis of representative early flowering T_1 *Arabidopsis* plants overexpressing *LLSEP3*. Approximately 10 μg of total RNA were loaded in each lane. Upper panel shows hybridisation with the *LLSEP3* probe. The blot was stripped and re-hybridised with the *NPTII* probe, shown in the lower panel. Lane 1 was loaded with RNA extracted from a non-transgenic plant, lanes 2 to 5 are from plants showing phenotypic alterations whereas lane 6 is derived from an *Arabidopsis* resembling its wild type.

Altogether, the sequence similarity between *LLSEP3* and E functional genes, the active expression through floral development and its persistence in mature floral organs, and the capability of promoting early flowering when overexpressed in the heterologous species *Arabidopsis* indicated that *LLSEP3* can potentially be the functional orthologue of *SEP3* in *Lilium longiflorum*.

Material and Methods

Plant material

Mature floral organs, developing buds and leaves were collected from lily (*Lilium longiflorum* Thunb.) plants growing under greenhouse conditions and immersed immediately in liquid nitrogen. Tissues were kept at -80^{o}C until use.

After seed vernalization for 3 days at 4^{o}C, wild-type *Arabidopsis thaliana* plants, ecotype Columbia (Col), were kept in a growth chamber under long day conditions at 23^{o}C until flowering. The first inflorescence stems were pruned to induce formation of lateral inflorescences for transformation.

LLSEP3 probe synthesis, cDNA library construction and screening

A homologous *SEP3* probe from lily for screening a cDNA library was produced by RT-PCR using total RNA derived from 1-cm floral buds and degenerated oligonucleotides designed from conserved regions between the end of the MADS-box and the carboxy-termini of *SEP3* homologous sequences available in the GenBank database. The first strand synthesis reaction was primed with an oligo-d(T)$_{12-18}$ supplied with the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen) and followed by a gene-specific amplification with the oligonucleotides 5'-CTC TAC GAG TTC TGC AGC-3' and 5'-GMT CCC ACA CYT GCT GC-3', as forward and reverse primers using an annealing temperature of 45^{o}C in the PCR. A 391-bp fragment was amplified, cloned into pGEM-T-easy vector (Promega, Madison, WI) and sequenced. High sequence homology could be observed between this clone and other putative *SEP3* homologues and therefore it was used as a probe for cDNA library screening.

A developing floral bud (1 to 3.5 cm) cDNA library from *Lilium longiflorum* was synthesised using the ZAP-cDNA Gigapack III Gold Cloning Kit (Stratagene, La Jolla, CA) with 5 μg of pooled poly(A)$^{+}$ RNA. Fragments ranging from 1 to 1.5 kb were selected for unidirectional insertion in Uni-ZAP XR phage vector. The cDNA clones were rescued into pBluescript SK^{+} by *in vivo* excision using the ExAssist helper phage. About 50,000 pfu were screened on nylon membranes at 60^{o}C with the 391-bp homologous probe.

Nucleic acids isolation and expression analyses

Total RNA isolation from lily tissues was performed according the method described by Zhou et al. (1999). Total RNA isolation from *Arabidopsis* inflorescences was accomplished with the Rneasy kit (Qiagen, Gmbh, Hilden, Germany).

For northern blots, 10 μg of RNA was prepared using a modified glyoxal/DMSO method (Sambrook et al., 1989) and run in 1.4% agarose gel made with 15 mM sodium phosphate buffer pH 6.5 and stained with ethidium bromide. RNA was transferred by capillary method to nylon membranes (Amersham, Buckinghamshire, UK) using 25 mM phosphate buffer pH 6.5 as transferring solution. As probe, a fragment of 497 bp from the 3' terminal part of *LLSEP3* was amplified by PCR using the oligonucleotides 5'-CAC ATC TTT GAA GCT AAT CCG-3' and 5'-AGA TAC ATA GAG CGG CTT GC-3' as forward and reverse primers, respectively. The product was purified by spin columns (Qiagen) and the probe was synthesised with the RadPrime DNA Labeling System (Invitrogen, Carlsbad, CA) using [^{32}P] dATP as radioactive label.

For loading control of RNA from lily, a fragment of 620 bp from the constitutive glyceraldehyde-3-phosphate-dehydrogenase (*GAPDH*) cDNA from *L. longiflorum* was amplified by RT-PCR using the degenerated primers (5'-GTK GAR TCN ACY GGY GTC TTC ACT-3' and 5'-GTR TGR AGT TGM CAN GAR ACA TC-3' for forward and reverse annealing, respectively), conceived from DNA sequence alignment of several *GAPDH* from monocot species available in the GenBank.

Arabidopsis northern blots were prepared as described above, using the same *LLSEP3* probe in transgenic plants of T_1 generation germinated on selection medium with kanamycin. For molecular confirmation of transgenic integration of the kanamycin resistance *NPTII* gene, we amplified a probe by PCR using the transformation vector as a template and the primers 5'-TGG GCA CAA CAG ACA ATC GGC TGC-3' and 5'-TGC GAA TGC GGA GCG GCG ATA CCG-3' for forward and reverse annealing.

Hybridisation was carried out at 60^{o}C for at least three hours with one hour pre-hybridisation in the buffer (10% dextran sulphate, 1% SDS, 1M sodium chloride and 10 μg mL^{-1} herring sperm). Washes were conducted at the hybridisation temperature for 20 min twice in 2X SSC, 0.1% SDS and once in 1X SSC, 0.1% SDS. Membranes were then exposed to phosphor image screen (Fuji Photo Film Co., Tokyo, Japan) overnight and the image was scanned and processed using TINA version 2.10 program (Raytest, Straubenhardt, Germany).

Sequencing and sequence analysis

Sequencing of the cDNA clones was performed using rhodamine dye (Applied Biosystems, Foster City, CA) and analyses were carried out with the DNASTAR software package for sequence assembly, conceptual translation and biochemical characterisation.

Multiple protein sequence alignment used the first 185 amino acids from the MADS-box (Rigola et al., 1998). Phylogenetic analysis was accomplished by the ClustalX program version 1.81 (Thompson et al., 1997) and the dendrogram was visualised by the TreeView software (Page, 1996). The GenBank accession numbers of the amino acid sequences used for these studies were: *AGL3* (AAB38975); *AP1* (CAA78909); *BoSEP1a* (CAD48303); *BpMADS1* (CAB95648); *CanMADS1* (AAF22138); *CAGL2* (AAF23363); *CDM44* (AAO22982); *CDM77* (AAO22983); *CDM104* (AAO22987); *CMB1* (AAA62761); *DAL1* (CAA56864); *DEFH49* (CAA64741); *DEFH72* (S71756); *DEFH200* (CAA64743); *DoMADS1* (AAF13260); *DoMADS2* (AAF13261); *DoMADS3* (AAF13262); *EGM1* (AAC78282); *EGM3* (AAC78284); *FBP2* (Q03489); *FBP4* (AAK21247); *FBP5* (AAK21248); *FBP9* (AAK21249); *FBP23* (AAK21254); *GGM9* (CAB44455); *GGM11* (CAB44457); *GhMADS1* (AAN15182); *GpMADS3* (BAA85630); *GRCD1* (CAC13148); *HAM137* (AAO18233); *HvMADS7* (CAB97353); *HvMADS9* (CAB97355); *MAGL4* (AAL08423); *MdMADS1* (AAC25922); *MdMADS3* (AAD51422); *MdMADS4* (AAD51423); *MTF1* (CAA11258); *NsMADS3* (AAD39034); *NtMADS4* (AAF76381); *OM1* (Q38694); *OsMADS1* (S53306); *OsMADS8* (*OsMADS24*, AAC49817); *OsMADS17* (AAF21900); *OsMADS45* (*OsMADS7*, AAC49816); *PaMADS1* (AAK50865); *PrMADS1* (AAD09206); *SaMADSD* (CAA69916); *SbMADS1* (AAB50187); *SEP1* (*AGL2*, AAA32732); *SEP2* (*AGL4*, AAA32734); *SEP3* (*AGL9*, AAB67832); *TAGL2* (AAM33104); *TaMADS12* (BAA33458); *TM5* (CAA43010); *VvMADS2* (AAM21342); *VvMADS4* (AAM21344).

Vector construction and *Arabidopsis* transformation

A binary vector derived from pBINPLUS (van Engelen et al., 1995) carrying the kanamycin resistance *NPTII* gene was used for harbouring the 35S::*LLSEP3* in sense and anti-sense orientation. *Agrobacterium tumefaciens*, strain AGL0, was prepared and transformed with the binary vector by electroporation according to Mattanovich et al. (1989). Wild type *Arabidopsis*, ecotype *Columbia*, was transformed by the floral dip method (Clough and Bent, 1998). T_1 progeny was selected on plates with 0.5X MS medium (Murashige and Skoog, 1962) supplemented with 50 μg mL^{-1}

kanamycin. Surviving seedlings were transferred to soil and acclimatised in greenhouse conditions. Self-pollinated T_2 offspring was sown directly on soil in order to confirm transgenic phenotype segregation.

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CHAPTER FOUR

Ascribing the function of *LLAG1* from *Lilium longiflorum* in the heterologous system *Arabidopsis thaliana*

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Abstract

Here, the functional characterisation of the gene *LLAG1*, from *Lilium longiflorum*, by means of complementing the *agamous* mutant of *Arabidopsis thaliana* is described and critically discussed. Drawbacks such as, on the one hand, the nature of the *AGAMOUS* gene, of which the loss of function induces sterility and, on the other hand, the unavailability of the defective *ag-1* allele in another *Arabidopsis* background than the *Landsberg erecta* ecotype, which is recognisably difficult to transform by our transformation method, led our efforts on functional analysis to be carried out with great difficulty. Even though we did not manage to complement the *AG* function with *LLAG1* in a defective *ag* genotype, we could observe clear floral homeotic changes in those *Arabidopsis* plants ectopically overexpressing *LLAG1*, which together with our data on sequence identities and expression profile described in the previous chapter of this thesis, indicated that *LLAG1* is a strong candidate to control the C function in *L. longiflorum*.

Introduction

In plants, the species *Arabidopsis thaliana* was chosen as the contemporary, most important model for genetic and developmental studies. Its short life cycle (around 6 weeks from germination to production of mature seeds), small size (15 to 20 cm height), natural self-fertilisation and large progeny (about 5,000 seeds per plant), small nuclear genome size (125 Mbp) distributed in only 5 chromosomes, and an easy transformation system made this species ideal to the quest for unravelling many plant biology aspects (Meinke et al., 1998; Meyerowitz and Somerville, 1994).

As a comparison, trumpet lily (*Lilium longiflorum*) can take up to two years to flower, has the largest genome in the plant kingdom, estimated to be 90 Gbp (more than 700 times larger than the *Arabidopsis* genome), located in 12 chromosomes frequently in multiple copies, and offers great recalcitrance to transformation. Notwithstanding the fascination for the beauty of their flowers, molecular biology studies in lily species have time and methodological constraints. A way to circumvent these problems is using model species such as *Arabidopsis* as a heterologous system, in order to assess gene function and other biological aspects of lily.

Studies on *Arabidopsis* started with Mendelian genetics (Laibach, 1943; Rédei, 1970; Koornneef et al., 1983) and is now culminating in the "-omics" era, in which its entire genome is already unveiled (*Arabidopsis* Genome Initiative, 2000; Meyerowitz, 2001; Somerville and Koornneef, 2002). The "-omics" approaches are capable to generate data in a high quantity and speed, but usually these data require further studies to validate the hypotheses generated with this information, especially concerning gene functions.

Good indications on gene functionality can be obtained by *in vivo* methods such as protein interactions in yeast hybrid systems (Causier and Davies, 2002; Immink and Angenent, 2002; Moon et al., 1999; Pelaz et al., 2001), fluorescence resonance energy transfer (FRET, Immink et al., 2002), gene overexpression and knock out (Hirschi, 2003) in homologous or heterologous species, nonetheless the final confirmation can only be accomplished by the introduction of a genetic function into a related defective genotype in order to recover a functional phenotype for the gene in study.

Complementation testing is an elegant and, in principle, simple way of assessing gene functions. Conceptually, it means that when two mutant genotypes with mutations in different *loci* are crossed, they will restore the wild-type phenotype in the F_1 siblings by complementing the defective allele of each other with the wild-type allele from the other parent (Figure 1).

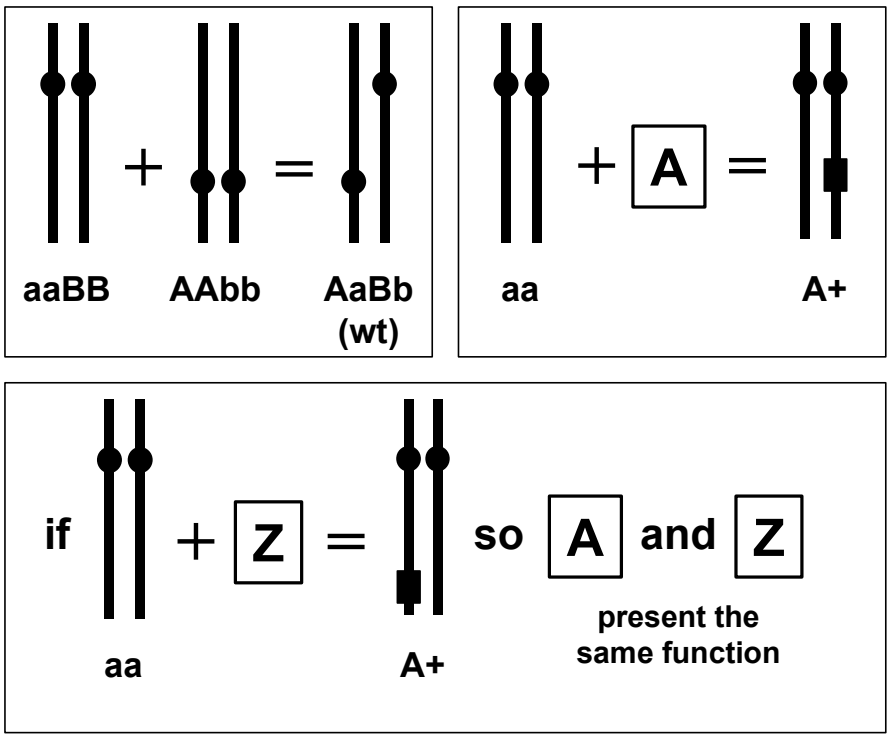

Figure 1. Diagram representing the genetic complementation test. Vertical bars denote homolog chromosomes in a diploid individual. Circles and letters in lowercase represent mutant recessive alleles whereas the absence of circles and the letters in uppercase represent the wild-type allele in a given *locus*. The traditional complementation test is shown in the left box on the top: crosses of two plants with defective mutations can restore the wild-type phenotype if the mutations are in different *loci*. The right box represent the alternative approach using a transformation system to introgress a functional gene in the defective genome. The squares correspond to the transgene whereas the signal + represents overexpression. In the lower box, the approach for characterising functional genetic homology is represented. If the integration of a given gene can restore the same phenotype as a confirmed functional gene does, one can say that both share the same function and are, therefore, functional homologues.

The advent of transformation systems brought the possibility of performing complementation tests without crossings between the defective genotypes, but instead it can be done with one defective genotype being complemented by an introduced functional gene via any transformation method available. Additionally, it allowed performing complementation tests in one species with genes originating from other species, in order to confirm functional orthologies.

Another important approach to confirm gene functionality is by creating dominant negative genotypes in which truncated gene fragments are introduced into the plant genome in order to express a defective protein that will block gene function and create a negative genotype for the gene in study in a dominant fashion (Chandler and Werr, 2003; Herskowitz, 1987; Mizukami et al., 1996).

Plant type II MADS-box genes show the MIKC structure with a highly conserved MADS-box (M), a weakly conserved intervening region (I), a conserved K-box (K), and a specific carboxy-terminal portion (C). The MADS domain is responsible for protein binding to the *cis*-regulatory elements of the DNA known as CArG box (Pollock and Treisman, 1991; Riechmann et al., 1996b), the I region is thought to act as a factor for selective dimerization (Riechmann et al., 1996a), the K domain controls protein-protein interactions (Ma et al., 1991; Fan et al., 1997), and the C-terminus is involved in specific transcriptional activation and protein interaction stabilisation (Cho et al., 1999). Overexpression of truncated MADS proteins were shown to induce dominant negative phenotypes in wild-type *Arabidopsis* (Krizek et al., 1999; Mizukami et al., 1996; Tzeng and Yang, 2001).

Lily (*Lilium longiflorum*) is one of the most important ornamental species worldwide and its flower development pathways are under investigation at the molecular level (Theissen et al., 2000; Tzeng and Yang, 2001; Tzeng et al., 2002; this thesis).

We endeavoured to assess the *LLAG1* function by performing complementation tests, and also inducing dominant negative *agamous*-like phenotype in the heterologous species *Arabidopsis*. Unfortunately, due to the nature of the *AGAMOUS* (*AG*) gene, which confers sterility when in homozygosity, and the transformation recalcitrance shown by the *Arabidopsis* ecotype *Landsberg erecta* (Clough and Bent, 1998), the objectives of this project are still under way.

Results

An *Arabidopsis* population containing the defective allele *agamous-1* (*ag-1*) was available in the *Landsberg erecta* (*Ler-0*) background. *AG* is involved in triggering the development of stamens and carpels, and also in flower determinacy. The *ag-1* allele, when in homozygosity, leads to homeotic alterations of the stamens into petals, and of the carpels into a new flower, conferring sterility to the plant. Therefore, the phenotypically indistinguishable *AG*/*ag-1* and *AG*/*AG* genotypes were used for *Agrobacterium tumefaciens* transformation in order to introduce *LLAG1*, from *Lilium longiflorum*, into the defective *Arabidopsis* genome. At the same time, wild-

type plants, *Columbia* (*Col-0*) ecotype, were also transformed by the same procedures and followed up to the T_2 generation. Figure 2 illustrates the transgenic *Arabidopsis* phenotypes obtained with *LLAG1* overexpression whereas Figure 3 shows their Southern and northern analyses, confirming transgene integration and its active transcription. The number of plants transformed, transformants recovered on selection medium and their respective phenotypes in T_1 generation are shown in Table 1.

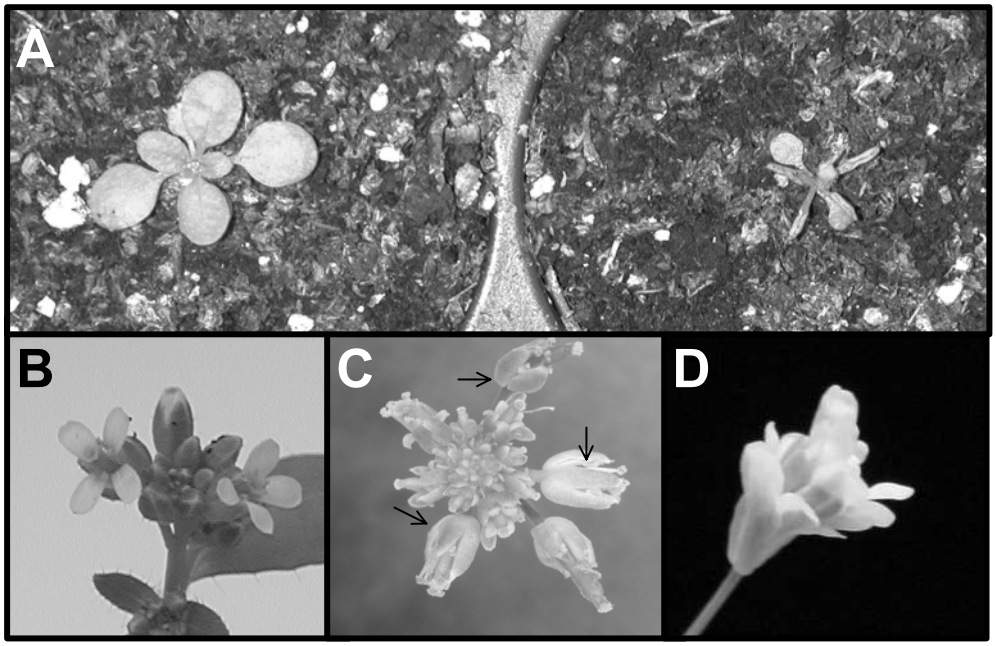

Figure 2. *Arabidopsis* phenotypes. (**A**) Sibling plants with *Col-0* background showing clear phenotypic segregation of wild-type phenotype (left) and overexpressing *LLAG1* (right) at 15 days after germination. (**B**) Inflorescence with *Col-0* wild-type flowers. (**C**) Flowers of an *LLAG1* transgenic *Ler-0* plant showing a strong *ap2*-like phenotype. Note the homeotic changes of petals into stamen-like organs (arrows). (**D**) A *Col-0 x Ler-0* flower with an *agamous*-defective phenotype showing the reiterated pattern $(sepals\text{-}petals\text{-}petals)_n$.

Ectopic expression of *AG* (or its functional orthologues) in the outer whorls of a flower induces homeotic changes of sepals into carpelloid organs and the petals into stamens, giving an *apetala2*-like phenotype, as stated by the ABCDE model of flower development, since *AP2* and *AG* have antagonistic functions (Coen and Meyerowitz, 1991). *AG* expression with strong constitutive promoters, such as the CaMV35S, leads to dwarf and weak plants, with few and curled leaves, early flowering, bumpy siliques, few seeds, and the aforementioned floral homeotic changes (Li et al., 2002; Mizukami and Ma, 1992; Rigola et al., 2001; Rutledge et al., 1998).

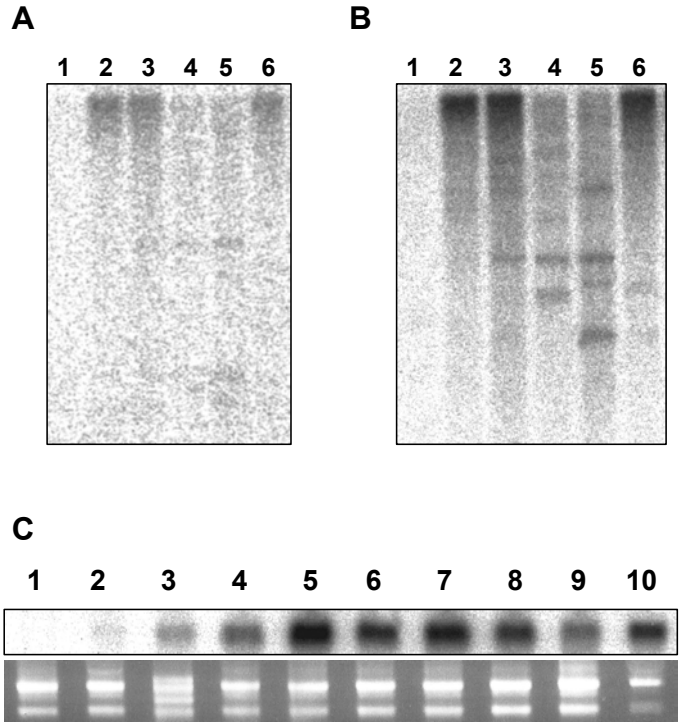

Figure 3. Southern and northern analyses of plants transformed with *LLAG1* (T_1 generation). Southern blot with genomic DNA derived from *Col-0 Arabidopsis* digested with *Hin*dIII showing multiple insertions of *NPTII* (**A**) and *LLAG1* (**B**). Lane 1 was loaded with DNA derived from a non-transgenic plant, whereas lanes 2 to 6 were derived from plants selected for kanamycin resistance. Plant 2 showed wild-type characteristics and plants 3 to 6 showed *ap2*-like phenotype. (**C**) Northern blot of flower RNA isolated from transformed *Ler-0 Arabidopsis* plants and probed with 3' portion of *LLAG1*, indicating its active transcription in transgenic plants. Lane 1 was loaded with RNA derived from a non-transgenic plant, whereas plants 2 showed a wild-type phenotype; plant number 3 showed a mild *ap2*-like phenotype, and plants 4 to 10 showed a strong phenotype. Lower panel shows RNA loading quantities manifested by ethidium bromide gel staining.

Table 1. Phenotypical segregation of *ap2*-like characteristics conferred by the overexpression of *LLAG1* in *Arabidopsis*.

	T0	T1				
Transformed background [a]	n [b]	WT	Mild *ap2*$^-$	Strong *ap2*$^-$	*ag*$^-$	n [c]
Ler-0 +/?	48	2	2	7	0	11
Col-0 +/+	16	32	13	4	0	49
Col-0/*Ler*-0 +/-	39	20	0	2	6	28

[a] *Arabidopsis* genetic background used for transformation: + means wild-type *AG* allele, - means the defective *ag-1* allele and ? means undetermined allele (since the plants presented wild-type phenotype, the defective allele was in the population). *Ler-0* is the *Landsberg erecta-0* ecotype, *Col-0* is the *Columbia-0*, whereas *Col-0*/*Ler-0* is a hybrid between the two ecotypes.

[b] indicates the number of plants used for floral dip transformation.

[c] indicates the total number of siblings studied in the population.

After the first transformation experiment, we realised that the *Ler-0* F_1 population was segregating the recessive homozygous *ag-1* mutants at much lower frequencies than expected from truly heterozygous parents, as ordered from the Seed Bank (Mendelian segregation ratio of 1 out of 4). Among 12 non-transgenic plants tested, only one presented the *ag-1* phenotype and, among the remaining 11 plants, only one plant showed segregation of the *ag-1* allele in the next generation. Hence, the seeds from this heterozygous plant were used for further experiments.

Molecular characterization of the allele *ag-1* was described by Lenhard et al. (2001) based on enzymatic digestion of a 317-bp PCR amplification product detailed in the Material and Methods section. In this system, the amplified fragment derived from the wild-type allele remains uncut, since there is no restriction site for the endonuclease used, whereas the defective allele is divided into two fragments of 297- and 20-bp because a point mutation created a *Hin*dIII site in the amplified region. Attempts were done to identify the differences between 317- and 297-bp fragments in agarose gels but, given the resolution of the technique, no satisfactory results could be obtained.

Arabidopsis ecotype *Col-0* has a higher competence for *Agrobacterium* transformation by the floral dip method than the *Ler-0* ecotype (Clough and Bent, 1998). In an attempt to increase the transformation frequency, the heterozygous *Ler-0* plant identified by progeny analysis was used as a pollen donor to cross with wild-type *Col-0* plants. Thirteen hybrid lines were analysed in the F_2 progeny to identify heterozygous genotypes, in which 8 plants showed segregation of the *ag-1* allele. Segregation of leaf morphology and inflorescence architecture was noticeable as well.

Plants derived from heterozygous plants (*Ler-0* x *Col-0 ag-1* / *AG*) were sowed and had their molecular genotypes assessed by fractionation of the restricted amplification products in a polyacrylamide gel, showing a clear difference between the uncut fragment of 317 bp and the restricted *ag-1*-derived fragment of 297 bp (Figure 4).

In order to increase the *ag-1* allele frequency in the T_1 generation and facilitate the identification of genotypes with *ag-1* in homozygosity complemented by *LLAG1*, only heterozygous plants were transformed with a binary vector containing the *LLAG1* cDNA. T_1 plants were selected on medium supplemented with kanamycin, transgeny was confirmed by Southern blot analysis, and *LLAG1* transcription was certified by northern blots (data not shown).

Alternatively, *LLAG1* function was tried to be assessed by inducing dominant negative *ag*-like phenotype in wild-type *Arabidopsis* ecotype *Col-0*. Expression of the truncated *LLAG1* protein without the MADS domain (*LLAG1*ΔMADS) was introduced in *Arabidopsis* via transformation with *A. tumefaciens*. Transgenic plants were selected on medium supplemented with kanamycin and seedlings were transferred to soil under standard conditions. Southern blot analysis confirmed the presence of the truncated transgene and its active transcription was verified by northern blots. No alterations could be observed in comparison to the wild-type floral phenotype among the 42 plants analysed in the T_1 and T_2 generations.

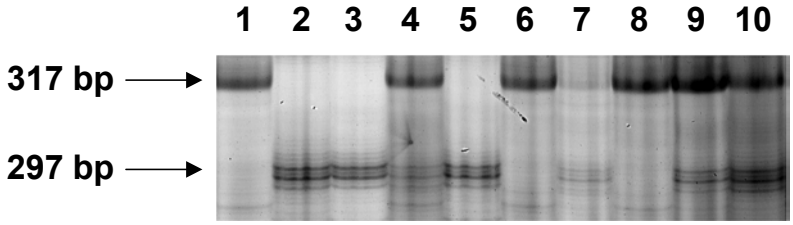

Figure 4. Segregation of *ag-1* allele in a population derived from a heterozygous *Arabidopsis* plant. Fractionation of a PCR fragment digested with *Hin*dIII in a polyacrylamide gel reveals two distinct sizes. Fragments of 317 bp are derived from the wild-type allele whereas the 297-bp fragments are originated from the mutant *ag-1* allele. Plants 1, 6, and 8 are homozygous for the wild-type *AG* allele. Plants 2, 3 and 5 are homozygous for the *ag-1* and showed the mutant flower phenotype. Plants 4, 7, 9 and 10 are heterozygous in this locus and presented wild-type flowers.

Discussion

Given the nature of the *AG* gene and the background in which the defective allele *ag-1* was available, complementation test of *LLAG1* in the heterologous model system *Arabidopsis* proved to be not a straightforward procedure.

Firstly, plants with *ag-1* in homozygosity could not be used due to the sterility conferred by the *AG* lack of function. Therefore, use of heterozygous *ag-1*/*AG* was compulsory in order to get, in a future generation, transgenic plants overexpressing *LLAG1* in a segregating *ag-1* homozygous genotype, which would be expected to show the *AG* function complementation, i.e. producing sexual organs in the flower.

Secondly, the *ag-1* allele was only available in the *Landsberg erecta 0* (*Ler-0*) background, which has been reported to have transformation rates at 10 to 100 fold lower than *Columbia* (*Col-0*) when using the floral dip method (Clough and Bent, 1998). Indeed, we found poor transformation rates with *Ler-0* plants whereas the *Col-0* background gave satisfactory number of overexpressing transformants under the same conditions.

Furthermore, the *ag-1* frequency in the *Ler-0* population was found to be much lower than expected if the source in the Seed Bank was truly derived from heterozygous plants instead of the seeds being derived from segregating plants being harvested in bulk. Once more, due to the nature of the *AG* gene, that confers sterility for its defective alleles when in homozygosity, bulk seed harvesting will continuously diminish the allele frequency in the population. Careful selection of heterozygous parents would lead to one fourth of the population with the defective allele in homozygosity, the same number of plants homozygous for the wild-type allele whereas half of the population would bear the heterozygous genotype. In a small experiment, we found that among 12 plants, only one showed the defective allele in homozygosity and only another one segregated the *ag-1* allele in a future generation, leaving 10 (5/6) plants putatively as wild types.

Molecular genotyping of *ag-1* is feasible, since a point mutation created a new *Hin*dIII restriction site in the gene. The method is based on amplification of a correspondent genomic fragment, its subsequent exposure to the endonuclease and fractionation in a gel. However, the primers used by Lenhard et al. (2001) amplified a product of 317 bp that would be cut in two fragments of 297 and 20 bp only if it was derived from the defective allele. Many attempts were performed to visualise the difference between the fragments of 317 and 297 bp in an agarose gel system but no satisfactory results were obtained. The obstacle could only be overcome by the

utilisation of a polyacrylamide gel, which, in the end, gave a fine and clear resolution between the undigested and the digested fragments.

It is clear, however, that *LLAG1* overexpression in wild-type *Arabidopsis* induces homeotic mutations in the floral organs, being capable to replace petals for complete stamens and inducing changes in sepal features, as predicted for a C functional gene by the ABCDE model and observed with several other C functional orthologues.

Although the finalisation of the complementation tests could not be accomplished within the time frame set to complete this work, sufficient evidence was obtained to strongly indicate that *LLAG1* can be indeed a C functional gene with a relevant role in establishing stamen and carpel identities in *Lilium longiflorum*. This is supported by close sequence similarities with other characterized C functional genes, its specific expression pattern and its capability of inducing the predicted homeotic mutations in *Arabidopsis* when ectopically overexpressed, as showed elsewhere in this thesis.

Material and Methods

Plant material, transformation and growth conditions

Seeds of an *Arabidopsis* line, ecotype *Landsberg erecta* (*Ler-0*), segregating the defective *ag-1* allele (TAIR stock number CS25) and a wild-type population in *Columbia* (*Col-0*) background were used in this study. Transformation of *Arabidopsis* plants was according to the floral dip method described by Clough and Bent (1998).

T_0 and T_2 *Arabidopsis* seeds were sowed directly on soil and, after a vernalization period of 3 days at 4°C, they were transferred to a growth chamber under long day conditions (16h light / 8h night) at 23°C. T_0 plants had their primary inflorescence stem pruned in order to produce higher number of flowers for transformation.

T_1 transformed seeds were placed onto 0.5X MS medium (Murashige and Skoog, 1962) with 0.8% agar and 50 mg L^{-1} kanamycin as a selection agent. Seed disinfection was accomplished by fumigation produced by the mixture of 100 mL 3% sodium hypochloride and 4 mL 37% hydrochloric acid in a desiccator for 4h. Vernalization followed as described above. After germination, seedlings were transferred to soil and kept in greenhouse under the same conditions detailed for T_0 plants.

Cloning of *LLAG1* into a binary vector and transformation

The plasmid pGD121 (Immink et al., 2002), derived from the binary vector pBINPLUS (van Engelen et al., 1995), and carrying a cauliflower mosaic virus (*CaMV*) *35S* promoter, a nopaline synthase (*NOS*) terminator, and a multiple cloning site between these regions was used to transfer *LLAG1* to plant cells via *Agrobacterium tumefaciens*.

For the complementation test in *Arabidopsis*, *LLAG1* was first cloned into the phagemid pBluescript SK^+ and selected from a cDNA library by hybridisation. A 1.2-kb fragment containing the whole *LLAG1* coding region was released by *Xba*I and *Xho*I restriction enzymes and ligated in a sense direction into the pGD121 opened with the same enzymes. *A. tumefaciens* competent cells (strain *AGL0*) were prepared and transformed by electroporation according to Mattanovich et al. (1989).

In order to produce a defective *ag*-like dominant negative phenotype in *Arabidopsis* with *LLAG1*, a truncated *LLAG1* fragment without the MADS-box (*LLAG1*ΔMADS) was amplified from the phagemid that carried the whole clone by PCR at an annealing temperature of 52°C with the forward primer 5'-ATG GTG TGA AAG GGA CTA TT -3' bearing an introduced start codon (underlined), and the reverse primer 5'-AAA GTC ACA AAA TAA TAC AGC-3'. The 705-bp fragment was ligated into TOPO TA 2.1 cloning system (Invitrogen, Carlsbad, CA). *LLAG1*ΔMADS was excised with *Bam*HI and *Xho*I restriction enzymes and inserted in the sense direction into pGD121 vector opened with the same enzymes. Preparation of *A. tumefaciens* competent cells (strain *C58C1*) and transformation were followed as described above.

DNA extraction and molecular genotyping

The segregating population of *ag-1* allele was molecularly genotyped to obtain heterozygous plants for this locus intending to increase efficiency in the complementation test.

Genomic DNA from *Arabidopsis* was extracted from mature leaves according to Liu et al. (1995). PCR-based genotyping was carried out as described by Lenhard et al. (2001). The *ag-1* allele has a point mutation that creates a *Hin*dIII restriction site and therefore this feature was used as a basis for molecularly genotyping the segregating population. Amplification of a 317-bp fragment from the *AG* gene was carried out with the AG1 primer set (5'-GGA CAA TTC TAA CAC CGG ATC-3' and 5'-CTA TCG TCT CAC CCA TCA AAA GC-3' as forward and reverse primers, respectively). PCR conditions were 95°C for 5 min, 40 cycles of 95°C for 1 min

denaturation, 55°C for 1 min annealing and 72°C for 1 min extension, and a final extension step of 10 min at 72°C. Five microliters of the PCR reaction were incubated at 37°C for 2h with 0.6 µL of restriction buffer and 4 units of *Hin*dIII.

The 317-bp PCR product derived from the wild-type *AG* allele was not digested by the restriction enzyme whereas the fragment derived from the defective *ag-1* allele was digested into two fragments of 297 and 20 bp. The difference between the 317-bp and the 297-bp fragment could not be visualised on a non-denaturing agarose gel. Digested PCR products were denatured and separated on a denaturing polyacrylamide gel and stained with silver nitrate according to Echt et al. (1996).

Analysis of transgenic plants

Transgenic plants were confirmed by PCR and Southern blots, and the transgene expression, by northern blots.

Total RNA from *Arabidopsis* was isolated from flowers using the Rneasy Plant Mini Kit (Qiagen, GmbH, Hilden, Germany). Ten micrograms of total RNA were denatured by the glyoxal/DMSO method (Sambrook et al., 1989), and run in a 1.4% non-denaturing agarose gel prepared with 15 mM sodium phosphate buffer pH 6.5. Blotting procedures followed the capillary method to transfer nucleic acids to a nylon membrane (Amersham, Buckinghamshire, UK) with 25 mM phosphate buffer pH 6.5.

Genomic DNA was isolated from leaves by the Dneasy Plant Mini Kit (Qiagen). Five hundred nanograms of genomic DNA were digested by restriction enzymes overnight with the appropriate buffer, run on a 0.8% agarose 1X TAE gel and blotted on nylon membranes by the capillary method, as described by Sambrook et al. (1989).

Hybridisation procedures were conducted identically for Southern and northern blots at 60°C for at least 3h with a previous 1h incubation in the hybridisation buffer with no probe (10% dextran sulphate, 1% SDS, 1M NaCl and 10 µg mL^{-1} herring sperm).

A 348-bp fragment corresponding to the specific 3' end of *LLAG1* was used as a probe and amplified by PCR with the primers 5'-GAT TGC TGA AAA TGA GAG G-3' and 5'-AAA GTC ACA AAA TAA TAC AGC-3' for forward and reverse annealing at 56°C. The product was purified by the QIAquick PCR Purification Kit (Qiagen). About 30 ng of the purified product was taken for radioactive labelling using [^{32}P] dATP with the RadPrime DNA Labeling System (Invitrogen). The probe was

purified from unincorporated nucleotides by the QIAquick PCR Purification Kit and introduced into the system after the pre-hybridisation incubation.

A 685-bp fragment of the *neomycin phosphotransferase* II (*NPTII*) gene, which confers resistance to kanamycin and is present in the binary vector as a selection marker, was used for probing northern and Southern blots. The gene fragment was amplified using genomic DNA in a PCR with 5'-TGG GCA CAA CAG ACA ATC GGC TGC-3' and 5'-TGC GAA TCG GGA GCG GCG ATAC CG-3' as forward and reverse primers and an annealing temperature of 60^{o}C.

RNA loading was checked by rRNA stained with ethidium bromide. Its gene fragment was obtained by PCR from DNA extracted from *Arabidopsis* leaves using in the oligonucleotides 5'-GCG GTT TTC CCC AGT GTT GTT G-3' and 5'- TGC CTG GAC CTG CTT CAT CAT ACT-3' as forward and reverse primers, respectively, using an annealing temperature of 65^{o}C.

Blotting washes were carried out at the same hybridisation temperature. Washing the membranes was conducted into the tube first briefly with a 2X SSC, 0.1% SDS solution and a second time for 20 min with fresh and identical solution. Blots were then transferred to another container in order to facilitate radioactivity measurement and washed once more with 1X SSC, 0.1% SDS for 20 min at the incubation temperature under agitation.

Membranes were exposed to Phosphor Imaging Screen (Fuji Photo Film Co., Tokyo, Japan) for 4h or overnight and image was developed by a related scanner system. Radioactivity signals were processed by TINA software version 2.1 (Raytest, Straubenhardt, Germany).

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CHAPTER FIVE

Transformation of *Lilium longiflorum* via particle bombardment and generation of herbicide-resistance plants

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Abstract

This work is a contribution towards the improvement of lily (*Lilium longiflorum* Thunb.) transformation procedures. A vector carrying the *Arabidopsis SUPERMAN* gene driven by the petunia flower-specific *FLORAL BINDING PROTEIN 1* promoter and the bialaphos resistance gene *phosphinothricin acetyltransferase* under the *CaMV35S* promoter was used to transform bulblet slices by particle bombardment. Our intentions were improving the transformation parameters for lily transformation in order to reach higher efficiency, and creating novel phenotypes in lily flowers using transcription factors originating from dicot plants. We were capable of obtaining transgenic lines expressing *in vitro* resistance to bialaphos. The transgenic plants were transferred to the greenhouse, grown and monitored for two flowering seasons. Flowers derived from these plants appeared normal and indistinguishable from wild-type flowers and the possible reasons for this are currently under investigation.

Introduction

Lily species (*Lilium* spp.) have a high aesthetic value as cut flowers or pot plants and are among the most important ornamental species worldwide since a long time.

There are many lily breeding programs in order to get novel phenotypes with higher market values. Until now these programs are being established based mainly on segregation and selection of natural variants, and interspecific hybridisation by wide crosses. Although still in the quest for rapid and inexpensive techniques for massive *in vitro* propagation, tissue culture is already well established for lily (reviewed by Aswath et al., 2001) and is used to assist in interspecific hybridisation, especially with embryo rescue, and to propagate commercial clones.

Despite the importance of lily as ornamental species, genetic manipulation at a molecular level has proven to be difficult and reports on lily transformation has been scarce. Few reports showed transformed products in lily species so far, most of them by means of particle bombardment as the DNA delivery system and at a low efficiency (Lipsky et al., 2002; van der Leede-Plegt et al., 1997; Watad et al., 1998; Zeng et al., 2001).

Langeveld et al. (1995) were the first to indicate that *Agrobacterium* was able to transform lily cells, although they could not regenerate a transgenic plant by this method. More recently, though, Mercuri and colleagues (2003) reported for the first time on a workable transformation system using this biological delivery system.

An optimised transformation protocol for lily and the availability of molecular tools, such as genes and promoters may contribute to speed up breeding programs in this species.

SUPERMAN (*SUP*) is a zinc-finger transcription factor involved in flower development of *Arabidopsis*, defining the boundaries between the third and the fourth whorls by limiting cell expansion and proliferation (Bowman et al., 1992; Kater et al., 2000; Sakai et al., 1995). Ectopic expression of *SUP* controlled by constitutive promoters in heterologous species such as tobacco (Bereterbide et al., 2001), petunia (Kater et al., 2000) and rice (Nandi et al., 2000) has been shown to lead to dwarf plants.

FLORAL BINDING PROTEIN 1 (*FBP1*) is a floral specific gene from petunia *(Petunia* x *hybrida*) which is involved in the ABCDE model for flower development. It is a B class gene, expressed in petals and stamens as shown in petunia flower development (Angenent et al., 1993). Expression of *SUP* under the control of the *FBP1* promoter resulted in reduced petal and stamen growth in petunia and tobacco

(Kater et al., 2000). We were aiming at new floral phenotypes in lily using this construct cassette.

Here we report the transformation of *Lilium longiflorum* by the particle bombardment method using a vector containing the *SUP* gene from *Arabidopsis* driven by the petunia *FBP1* promoter and the bialaphos resistance gene *PAT* under the constitutive *CaMV35S* promoter. Transgenic plants showed *in vitro* resistance to the herbicide in the culture media and were transferred to greenhouse for flower morphology evaluation. They did not show alterations when compared to wild-type plants.

Results

Slices of *in vitro* cultivated bulblet scales from *Lilium longiflorum* were placed for one week on callus induction medium and bombarded with a plasmid harbouring the *CaMV35S*::*PAT* and the *FBP1*::*SUP* cassettes. Quantification of the transformation events and transient gene expression were tracked by parallel bombardments using a plasmid with the *CaMV35S*::*GUS*.

About 900 explants were exposed to the plasmid containing *SUP* under the petunia floral-specific promoter *FBP1*. These dishes were divided into 2 treatments: one shot at 1100psi (1x1100) or three consecutive shots at 1800psi (3x1800). The main differences between the bombardment protocol described by Watad et al (1998) and the protocol we used here are visualised in Table 1.

Transient *GUS* expression was measured by counting the number of blue spots per explant 5 days after bombardment. A considerable difference could be observed between the treatments (Figure 1) with double the number of transient events observed in the 1x1100 compared to the 3x1800 treatment.

The construct containing the *FBP1*::*SUP* cassette could not be accessed for transient expression because it does not contain a reporter gene. Explants were maintained in darkness for 4 weeks and then transferred to dimmed light conditions (ca. 30 μE s^{-1} m^{-2}) for an additional week before they were exposed to direct light (ca. 60 μE s^{-1} m^{-2} with a photoperiod of 16 h). Addition of selective agent to the culture medium started one week after bombardment throughout the subsequent steps.

Out of about 900 explants bombarded, 3 Basta® resistant plants were recovered and, despite the higher efficiency on transient expression of the 1x1100 treatment, these plants originated from the 3x1800 treatment.

Table 1. Differences between the method for *Lilium longiflorum* transformation via particle bombardment as described by Watad et al. (1998) and the protocol used in this report.

Parameters	Watad et al. (1998)	This report
Bombarded tissue	Embryogenic callus	Bulblet scale slices
plasmid purification	Cesium Chloride	Column (Qiagen)
DNA carrier	Tungsten	Gold
DNA amount/bombardment	2 μg	~0.8 μg
Target distance	6 and 9 cm	9 cm
Shooting times and pressure	1x900, 1x1100 and 1x1500psi	1x1100 and 3x1800psi
Selection agent (Basta)	Added after sterilization	Added prior to sterilization
Transfer to light	2 days after bombardment	4 weeks after bombardment
Transgenic plant recovery	19 plants/1800 calli	3 clones/900 bulblet slices

Plants were tested for genome integration of *PAT* and *SUP* genes. Attempts to perform Southern analysis of the transgenic lily genomes were done but no conclusive results could be obtained. Alternatively, the presence of these genes was assessed by PCR. All three regenerated plants growing on 2mg L^{-1} Basta® were positive for the presence of the *PAT* gene, in accordance with their observed resistance. Two of them, clones 1 and 2, were positive for *SUP* (Figure 2). Importantly, clones 1 and 2 were derived from the same bombardment dish. Final evidence for their individual transgenic character requires Southern analysis, however, because this technique was not applicable for lily, this question remains unanswered. All clones showed a normal phenotype during the vegetative phase, although the clone number three showed a slightly weaker phenotype, with fewer and thinner roots under *in vitro* conditions.

Each regenerated clone was first propagated *in vitro*, vernalized for at least 90 days at 4°C and then transferred to greenhouse conditions for acclimation in order to induce flowering. These plants were monitored for two growing seasons. The clone that showed absence of *SUP* (clone three) flowered normally in both seasons, whereas plants derived from the other two clones flowered aberrantly in the first year, stopping floral bud development at early stages, but produced normal flowers during the second season.

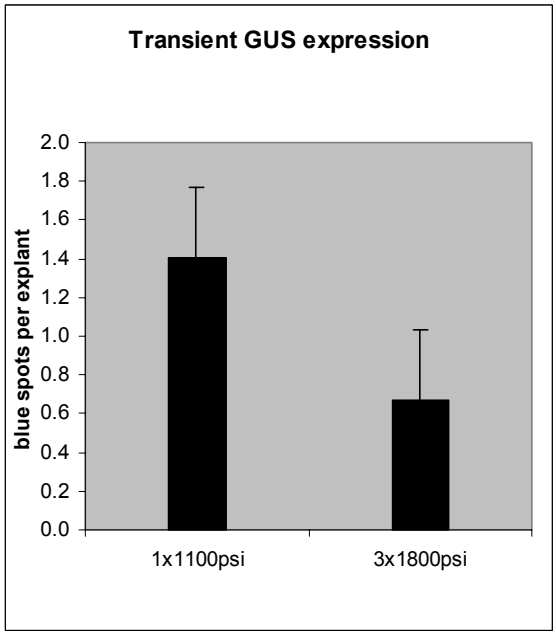

Figure 1. Transient *GUS* expression at 5 days after bombardment. A single bombardment at 1100psi (1x1100psi) gave double the number of transient expression events compared to the treatment with 3 shots at 1800psi (3x1800psi). Vertical bars show standard error.

SUP and *PAT* expression was examined by northern analysis of leaves and floral meristems. The preliminary transcription profile is shown in Figure 3. Northern blot analysis using RNA derived from leaves of bialaphos resistant plants shows expression of *PAT* and absence of its transcription in a non-transgenic plant (Figure 3a). Transcriptional evaluation of clone 2 floral meristem collected in the first flowering season shows expression of *SUP* whereas it is not visualised in a non-transgenic floral meristem (Figure 3b), indicating that the dicot *FBP1* promoter was actively coordinating *SUP* expression in clone two. Transcription of the constitutive *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) gene was used for comparison of RNA loading in the northern blots.

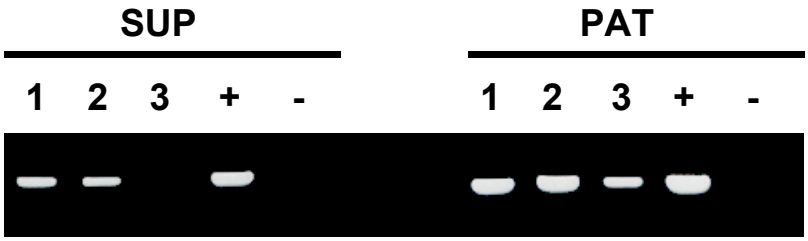

Figure 2. PCR amplification of inserted genes. *PAT* gene fragment was amplified in the three recovered clones, whereas the *SUP* gene is present only in two of the clones. Positive control (+) was carried out with the plasmid used for transformation, and wild-type genome of *Lilium longiflorum* was used as a negative control (-) of PCR reaction.

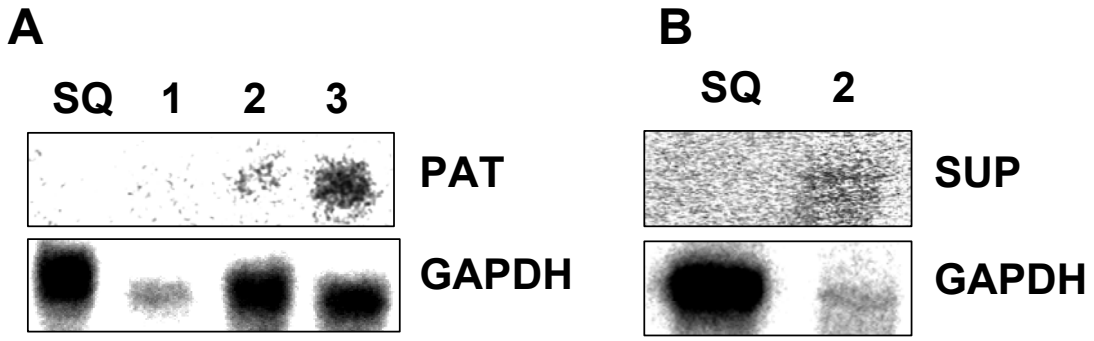

Figure 3. Northern analysis of gene expression in transgenic plants. (**A**) Total RNA isolated from leaves derived from transgenic clones (1, 2 and 3) and a non-transgenic plant (SQ) hybridised with the *PAT* probe. (**B**) Total RNA from floral meristems of clone 2 and from a non-transgenic plant was hybridised with a *SUP* fragment. Loading control is shown by hybridisation with the constitutive *GAPDH* gene visualised in the lower panels.

Discussion

Although lily (*Lilium longiflorum*) transformation mediated by microprojectile bombardment is feasible, it has always shown low efficiencies. Concomitant with our efforts of setting up an optimised protocol for lily transformation based on bombardment, Watad et al. (1998) obtained transgenic lily plants by bombarding morphogenic calli derived from bulblet scales. In fact, the very first transgenic lily was produced in our lab a few years before, using a pollen-mediated transformation method in which bombarded pollen was used to pollinate non-transgenic flowers (van der Leede-Plegt et al., 1997). More recently, another report was published on *L. longiflorum* transformation using a defective virus gene under the *CaMV35S* promoter to induce resistance against the cucumber mosaic virus (CMV) (Lipsky et al., 2002).

These results confirm the feasibility of lily transformation, although the scarceness of reports for this species also indicates the difficulties involved in the process.

In our studies, we recovered three transgenic clones, strikingly all of them from the 3x1800 treatment, when it was evident that there was a much higher transient *GUS* expression with the 1x1100 procedure. It is possible that, the number of transgenic clones obtained in the experiment may not be statistically significant to consider the 3x1800 treatment as the most adequate. Alternatively, the transient *GUS* expression may not reflect the permanent events in the genome, and if the DNA integration happened at a higher frequency in the 3x1800 treatment, the regeneration of three transgenic clones in this treatment and none with the 1x1100 treatment, would be understandable. None of these hypotheses can be refuted at this moment and further investigation is needed to clarify which parameters are more efficient to transform lily.

Recent experiments obtained in our lab indicated the importance of the callus induction period and the selective agent. Now, a reproducible method is available albeit with still low frequencies.

Confirmation of transgene integration into the lily genome could not be obtained by Southern blots. Despite a few reports showing Southerns in lily (Patil et al., 1995; Mercuri et al., 2003; Wang et al., 1999; Watad et al., 1998), this method is still very difficult in the species and, in addition to us, other scientists have reported difficulties with this procedure (Kanno et al., 2003). The main constriction is usually attributed to the colossal lily genome size, of around 90 Gbp, which is about 10 times the size of the human genome and about 700 times larger than the *Arabidopsis* haploid genome.

In our efforts to optimise the genomic DNA amount in PCR reactions, consistent results could only be obtained when using around 500 ng DNA per 50 μL PCR reaction. This can also be explained by the genome size of lily, which requires a higher mass of DNA to obtain the same number of target sequences present in a model species, such as in *Arabidopsis*. The same DNA amount was used by Watad et al. (1998) for transgene confirmation by PCR, suggesting that our results are not because of local experimental conditions, but due to intrinsic lily biological features.

Here, we also report the transformation of lily using a tissue-specific promoter from a dicot species (petunia) to drive the transcription of a floral gene from *Arabidopsis*. Studies of dicot promoter activity in a monocot species are of great biological relevance, and, even more intriguing is to understand gene activity across plant classes, such as a gene from a dicot species involved in flower development being ectopically expressed throughout the B domain of the lily flower.

The exact location of B-type gene promoter activity in lily is an interesting question. According to the ABCDE model for flower development, which was firstly conceived by analysing flowers from dicot species, such as *Arabidopsis* and *Antirrhinum*, the B domain would be placed in the petals and stamens domains of a flower (Coen and Meyerowitz, 1991). Considerations have been made about the B function in lily and tulip flowers, and some clues lead to think that in these species, the B function also commands the development of the first whorl organs. The first indication is that the first and second whorls in lilies and tulips show similar structures, forming a perianth of petal-like organs generally called tepals, instead of the distinct sepals and petals normally found in dicot species. This was even more evident when two mutant forms of tulip were found in which the tepals were converted into sepal-like organs in both whorls (van Tunen et al., 1993) indicating that the same factor was responsible for giving both these organs the petal appearance. The most obvious candidate for producing such a change would be a gene with the B function, according to the ABCDE model for flower development and the modified ABCDE model as proposed for Liliaceae species (van Tunen et al., 1993; Theissen et al., 2000; Kanno et al., 2003).

In order to get more information on this alternative model for flower development, it was tempting to introduce a transcription factor that limits cell expansion and proliferation (*SUP*) under expression control of a dicot floral gene promoter (*FBP1*). It was believed that an enhanced presence of such a transcription factor would lead to changes in floral morphology. Unfortunately, no altered phenotype was observed in transgenic clones in the second flowering season, although we could demonstrate expression of *SUP* gene in floral meristems of clone 2 plants under a dicot floral gene promoter. A complete analysis of plants from the remaining clones is still under way. However, it can be speculated that the reason why transgenic lily failed in showing altered flower morphology was because the number of transgenic clones obtained was too low to relevantly demonstrate a biological event. Alternatively, it can be that the dicot elements introduced (*SUP* gene or *FBP1* promoter) did not work efficiently to result in floral morphological changes.

Fundamental questions on how dicot transcription factors and gene promoters work in a monocot species, together with the necessity of improving the lily transformation protocol, drove the research we report in this chapter. The answers would help to better understand the reproductive biology and floral gene regulation in lily and monocot species as a whole, and provide new tools for molecular ornamental breeders.

In any case, the experiment resulted in three transgenic plants, which is a too limited number of plants to draw final conclusions on biological mechanisms, given the complexity of the cellular system and the many other parameters that may affect the results.

Material and Methods

Plant material and tissue culture. Inner bulblet scales from *Lilium longiflorum* Thunb. cv. Snow Queen established under *in vitro* conditions were sliced in transversal segments of 1 mm thick and 3-5 mm in length. Sixty explants per Petri dish were put onto callus induction medium (CIM, 1/2 strength MS salts with vitamins [Murashige and Skoog, 1962], 3% sucrose, 0.1 mg L^{-1} BAP and 1 mg L^{-1} picloram) for one week in the dark at 24°C. Before bombardment, these explants were transferred to fresh CIM and concentrated in the centre of the dish in a 5cm-diameter shooting target area.

Just after bombardment, explants were spread again and kept on the same medium for an additional week at the same pre-bombardment conditions. After this period, explants were transferred to CIM supplemented with the selective agent (Basta® 2 mg L^{-1} supplemented prior to media sterilisation) for two weeks. The explants were then transferred to regeneration medium (full strength MS medium with vitamins, 3% sucrose and 0.1 mg L^{-1} NAA) supplemented with the selection agent and kept in the dark for one week and then transferred to dimmed light conditions (under light with a white towel covering the dishes, ca. 30 μmol m^{-2} s^{-1}) for one week. After that, they were exposed to direct light (ca. 70 μmol m^{-2} s^{-1}).

Subculture was carried out every 4 weeks on the same medium until the regenerated plants with bulblets and leaves were recovered and put on propagation medium (1/2 strength MS salts and vitamins with 5% sucrose) supplemented with the selection agent.

Transformation vector and bombardment procedures. The *FBP1*::*SUP* cassette was inserted in the pBluescript KS+ plasmid harbouring the bialaphos resistance *phosphinothricin acetyltransferase* (*PAT*) gene under the constitutive CaMV35S promoter for selection. This vector was referred to as pB-SUP and the final size of this construct was 6.8kb. As a control for the transformation events, a 7.4-kb plasmid (pPG5) harbouring the β-glucuronidase (*GUS*) marker gene under the CaMV35S promoter was used for transient expression analysis and transformation efficiency measurement. DNA was purified by columns using the Plasmid Maxi Kit (QIAGEN, GmbH, Hilden, Germany).

The transformation experiment used about 900 explants divided over 15 dishes. The bombardment was carried out using the Biolistics PDS 1000/He system (Sanford et al., 1993) using 1.0 μm gold particles (Bio-Rad, Veenendaal, the Netherlands) as a carrier. Three milligrams of particles were coated with 5 μg of plasmid DNA (in a 60 μL solution) and 7.5 μL was used for each bombardment. Experiments were set up with 1 shooting at 1100 psi (treatment 1x1100) on about 400 explants or 3 shootings at 1800 psi (3x1800) on around 500 explants using a target distance of 9 cm. Additionally, 50 explants per treatment were bombarded with the control plasmid, pPG5.

Selection and identification of transgenic plants. Plants were kept on propagation medium supplemented with 2 mg L^{-1} Basta® to select for plants constitutively expressing the *PAT* gene. The regenerated plants were tested for the presence of the transgenes by PCR. Additionally to the Basta® resistance, the presence of *PAT* was identified by a PCR product amplification using the oligonucleotides 5'-GAT TAG GCC AGC TAC AGC AGC-3' and 5'-CCT TGG AGG AGC TGG CAA CTC-3' as forward and reverse primers, respectively, at an annealing temperature of 65°C. The presence of *SUP* in the genome was recognised by the amplification of a 613-bp fragment using the gene-specific primers 5'-ATG GAG AGA TCA AAC AGC-3' (forward) and 5'-TTA AGC GAA ACC CAA ACG-3' (reverse) using 55°C for the annealing step. The genomic lily DNA was extracted using the method described by van Heusden et al. (2000) and 500 ng were used in the PCR reaction.

Explants bombarded with pPG5 were assayed for transient β-glucuronidase activity after 5 days after bombardment using standard conditions (Jefferson et al., 1987).

Transgenic plants were kept *in vitro* until their bulblets were 2-3 cm diameter to be vernalized and transferred to soil under greenhouse conditions. These plants were followed by 2 flowering seasons under standard environmental conditions for flowering production in the greenhouse.

Assessing transgene expression. Floral meristems and leaves derived from transgenic lily plants growing in greenhouse conditions were harvested. They were immediately frozen in liquid nitrogen and kept in –80°C for further analysis. Total RNA was extracted by the Zhou et al. (1999) method. Northern blot, probe synthesis and purification, and hybridisation procedures were as described elsewhere in this thesis.

Constitutive transcription, for RNA loading control in northern blots, was

assessed through the expression of the *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) gene, that participates in the glycolysis pathway. A 620-bp fragment of the lily *GAPDH* was amplified by RT-PCR using the degenerated primers (5'-GTK GAR TCN ACY GGY GTC TTC ACT-3' and 5'-GTR TGR AGT TGM CAN GAR ACA TC for forward and reverse annealing, respectively), cloned in pGEM-T-easy vector (Promega, Madison, WI), sequenced and used as a probe.

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CHAPTER SIX

FLORAL HOMEOTIC MUTANTS IN LILY:

the double flower and *festiva* phenotypes

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Abstract

Homeotic changes in floral organs of lily (*Lilium* spp.) are described in this chapter. Usually, lily flowers show similar organs in their first and second whorls called tepals. They constitute the appealing colourful features determining flower appearance. Stamens and the carpel appear as the third and fourth whorls, respectively. A double lily flower shows replacement of stamens by tepals and of its carpel by a new flower in a reiterated manner, similar to what is seen in the *agamous* mutant of *Arabidopsis*. A novel floral phenotype of lily, denominated *festiva* here, has never been reported in other species so far and shows a complete homeotic change of stamens into tepals, but keeps the carpel identity. We tried to explain these phenotypes taking into consideration all the evidence on the genetic mechanisms involved in flower development gathered over the last 15 years. This work launches challenges and encouragement for exploiting the molecular mechanisms involved in flower development of lily.

Introduction

The economical importance of lily species (*Lilium* spp.) lies in their attractive flowers. Lily flowers already present a huge phenotypic variation in their colours, patterns, shapes and sizes. Even though, novel and aesthetically interesting phenotypes are welcome in the market, since novelty is a highly priced commodity in the ornamental business.

Homeotic changes in floral organs are easily noticed and could give rise to novel varieties. Double flowers are the result of homeotic mutations of stamens into petals and, frequently, of carpels into a new flower. This phenotype is found in a range of species, including lily. Other phenotypes can also be found in lily, such as partial homeotic changes of stamens into petal-stamen hybrid organs or a complete change of stamen into petal but keeping the carpel identity unchanged (**Figure 1**).

Many of the homeotic changes observed are the result of environmental stresses, such as high temperatures, being characterized as epigenetic events that do not segregate according to the Mendelian laws (**Figure 1b**). Other homeotic identity changes, however, are genetic alterations originated from loss-of-function of important genes that will segregate the alterations in future generations, if their sexual functions are not compromised in the mutant, as it is in complete double lilies (**Figure 1d**).

The ABCDE model for flower development was set up based primarily on dicot model species, such as *Arabidopsis*, *Antirrhinum* and *Petunia* (Coen and Meyerowitz, 1991; Colombo et al., 1995). It explains the conversion of a vegetative meristem into reproductive structures as the act of specific genetic regulatory functions working in an overlapping manner.

This model can be applied to lily flower development and can explain the formation of double lilies. However, we found a novel phenotype in lily with a complete transformation of stamens into tepals, but keeping the wild-type identity of carpels, which seems not readily explained by the current ABCDE model.

Here, we present lily flower development in the light of an adapted ABCDE model. We also introduce homeotic floral phenotypes and suggest hypotheses on the molecular genetic mechanisms that would bring them up.

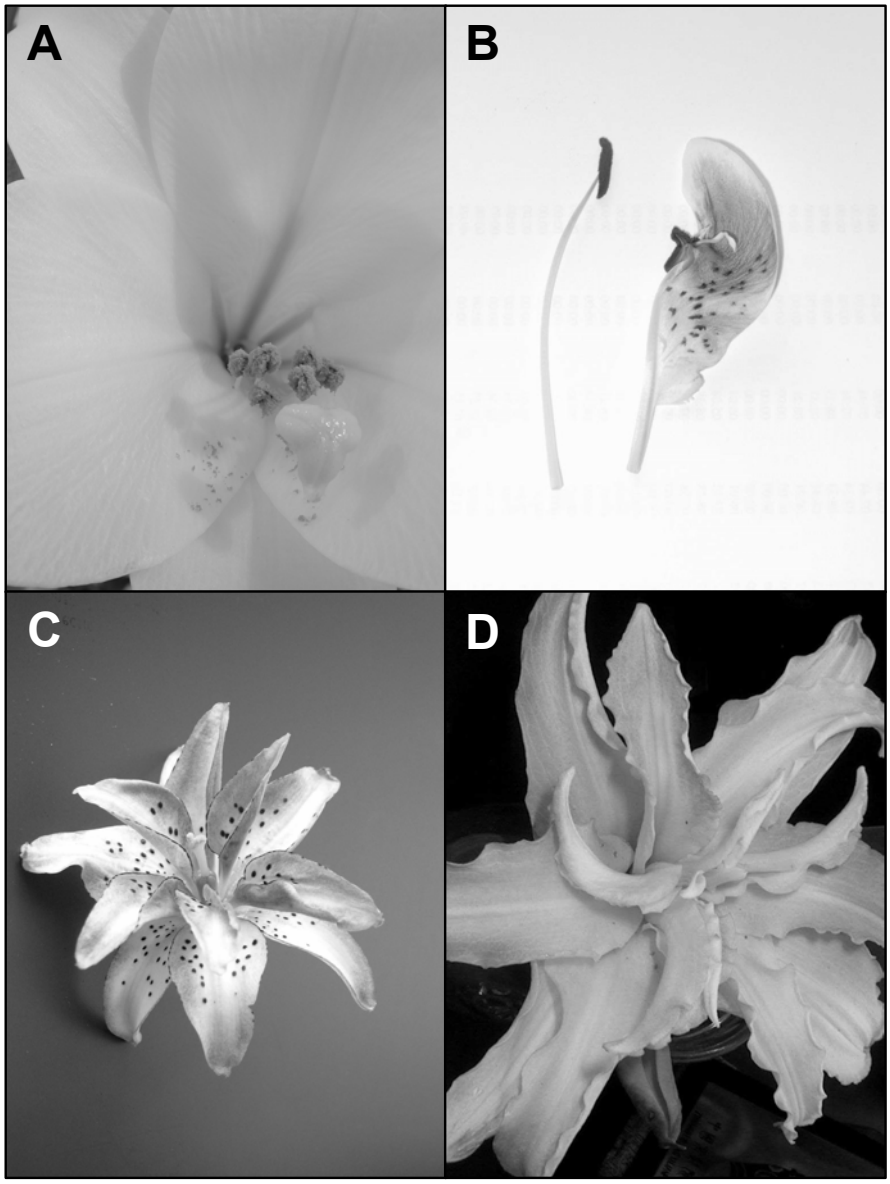

Figure 1. Floral phenotypes in *Lilium* spp. **(A)** Wild-type flower, with six tepals distributed in the two outer whorls, six stamens and a carpel in the inner whorls. **(B)** Wild-type stamen (left) next to a hybrid tepal-stamen organ (right), arisen due to physiological stress induced by heat. **(C)** *festiva* phenotype with complete homeotic change of every stamen to a tepal and showing a normal carpel development. **(D)** Double flower showing complete replacement of stamens by tepals and of its carpel by a new flower in a reiterated manner.

The modified ABCDE model for lily flower development

In flowers from *Lilium* species the two outermost whorls are composed of similar petal-like organs designated tepals. Stamens are distributed in two whorls (designated here as just one whorl, the third, for the sake of simplicity when comparing

to dicot model species). A carpel is regarded as the innermost whorl of lily flowers.

In our efforts to study lily floral development at the molecular level, we also attempted to isolate a gene with an A function. In order to generate a homologous probe for cDNA library screening, we tried amplifying a homologous fragment using degenerated primers conceived from *AP1* monocot sequences (Theissen et al., 2000). Contrary to our successes in amplifying a putative B (*PI*-like, data not shown) and of E-type gene fragment (*SEP3*-like), we were unable to amplify a putative A-type fragment. Therefore, this function still remains a mystery in lily species, since no orthologues with an A function have been reported by others as well.

The presence of tepals in the first whorl of lily flowers can be explained by the extension of the B and E functional domains towards the outermost whorl. If so, the loss-of-function of a B functional gene would lead to homeotic conversions of tepals in both outer whorls into sepal-like organs. This phenomenon was indeed observed in tulips, which belongs to the Liliaceae family, corroborating the modified ABCDE model in this plant family (van Tunen and Angenent, 1993).

In this way, the B function presents a special character in Liliaceae flower development and its candidate genes have already been object to scientific study. The first gene belonging to the ABCDE model from *Lilium* to be functionally investigated was a B-type orthologue of *APETALA3* (*AP3*) designated *LMADS1* (Tzeng and Yang, 2001). Strikingly, instead of showing a similar expression level of this gene in tepals from both whorls, *LMADS1* showed a stronger transcription activity in the second whorl organs and no protein was detected in the first whorl. This result raised uncertainties on the developmental identity of organs from the first and second whorls.

Another deviating feature found in B functional genetics of Liliaceae species is the facultative homodimerization of *PISTILLATA* (*PI*) orthologues from *Lilium regale* *LRGLOA* and *LRGLOB* whereas B type proteins from dicot species show obligate heterodimerization with other B type proteins (Winter et al., 2002). The same phenomenon was observed in *Tulipa gesneriana*, with the obligate heterodimerization of the B types *TGDEFA* and *TGDEFB*, but with the facultative homodimerization of the B type *TGGLO* (Kanno et al., 2003). These results support the dislocated B function in Liliaceae species, since the absence of *AP3* orthologues in the first whorl organs may be functionally circumvented by the alternative *PI*-like protein homodimerization.

We isolated and studied the molecular and functional characteristics of a putative C functional gene from *Lilium longiflorum*, *LLAG1*, described in the second chapter of this thesis. Functional analysis was carried out in the heterologous species

Arabidopsis, giving the expected homeotic changes, including a complete homeotic conversion of petals into stamens.

A putative D functional gene from *Lilium longiflorum* was also recently characterized (Tzeng et al., 2002). This function is responsible for ovule development in dicot species. Interestingly, when overexpressed in *Arabidopsis*, the lily D-type gene did not induce ectopic ovule formation, but instead, produced floral homeotic mutations similar to what a C-type gene would do, such as *AGAMOUS*.

The more recently characterized function in the ABCDE model, the E type, was also analysed in lily during our studies (chapter four). We were able to isolate and perform a molecular characterization of a putative E functional homologue from *Lilium longiflorum*, *LLSEP3*. Overexpression of this gene in *Arabidopsis* showed early flowering phenotype and no homeotic mutations, as observed when the *SEP3* is overexpressed in *Arabidopsis*. Additional studies must be carried out to establish the function of *LLSEP3*, such as its protein-protein interactions and capability of inducing leaf conversion into petal when overexpressed together with A and B genes. However, its sequence identities and ability of inducing early flowering are good indications that *LLSEP3* has indeed the E function in lily.

The C function and the double lily

The molecular elucidation of the mechanisms involved in the development of double flowers was started in *Arabidopsis*, in which a T-DNA insertion in a certain gene produced its loss of function, enabling to trace the responsible gene for the phenotype (Yanofsky et al., 1990). The *Arabidopsis* double flower phenotype showed conversion of stamens into petals, and the replacement of the carpel by a new flower, in a reiterated fashion. This phenotype was named *AGAMOUS* (*AG*) (from the Greek: *a* = not, and *gamous* = fecundate; due to its lack of sexual organs). The gene was recognised as a transcription factor and, together with three other genes, established the MADS-box gene family, which currently encloses more than 450 genes from many species (for a list see http://www.mpiz-koeln.mpg.de/mads/madslist.html).

In the *agamous* mutant, the homeotic conversion of stamens into petals is explained by the ABCDE model by the antagonism between A and C functions (Coen and Meyerowitz, 1991). If there is loss of C function, the A function takes over its domain, and vice versa. Stamens, which development is dictated by B+C+E functions, in the *agamous* phenotype are comprehensively replaced in the third whorl by petals, which is the result of A+B+E functions. Additionally, in this mutant, the flower becomes indeterminate, with its fourth whorl being replaced by a new flower, in a

reiterated way.

The emergence of this phenotype could also involve *AG* interactors that normally activate its expression, such as *WUSCHEL* (*WUS*) or *LEAFY* (*LFY*). Failure of normal expression of such genes in the floral meristem would lead to abnormal *AG* expression, and consequently to the aberrant floral phenotype (Roeder and Yanofsky, 2001).

Similar *agamous* homeotic changes were found in lily flowers (**Figures 1d, 2b**), showing both floral homeotic changes and loss of floral determinacy, indicating that the C function is conserved in Liliaceae species and that probably the same gene is, ultimately, dictating both characteristics, the stamen and carpel identities, and the floral determinacy.

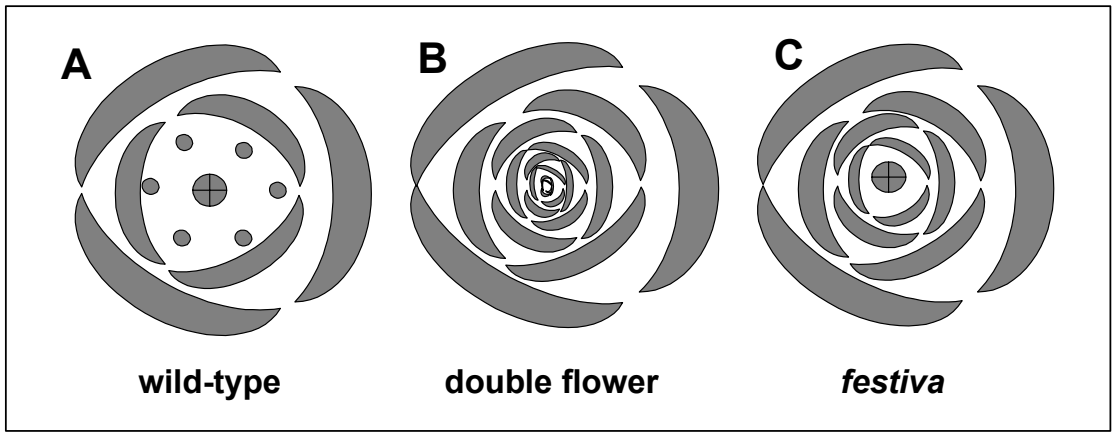

Figure 2. Diagram representation of lily floral phenotypes. **(A)** Wild-type flower, with tepals in its two outer whorls, stamens in the third whorl (circles) and a carpel in the inner whorl (crossed circle in the centre). **(B)** Double flower with tepals replacing every floral organ in a reiterated manner. **(C)** *festiva* phenotype, showing replacement of stamens by tepals and keeping its carpel identity.

The novel lily phenotype challenges the ABCDE model

Due to the overlapping feature of the ABCDE model, any loss in a given function of the model results in homeotic changes in organs of two adjacent whorls (except for the D and E functions). Whereas the wild-type lily flower presents the pattern [tepals-tepals-stamens-carpel], from outer to innermost positions, the loss of function of an A-type gene would give the pattern [carpels-stamens-stamens-carpel], the lack of a B function would lead to [sepals-sepals-carpels-carpel], mutation in the C function would be noticed by the reiterated pattern [tepals-tepals-tepals-new flower],

as visualised in double flowers.

Many studies on the genetics of flower development have been carried out in *Arabidopsis* and other model species creating an extensive catalogue of homeotic mutants, showing wide allelic series of floral homeotic genes in many cases.

Strikingly, we found a novel homeotic mutant phenotype in lily flower that has not been reported in other species. The phenotype, with normal tepals in the first two whorls, presents a complete homeotic conversion of its stamens into tepals, as it is seen in the *agamous*-like mutant, but shows a completely normal carpel development in its innermost whorl, differing from the C-type loss of function. We called this phenotype *festiva* (from the Latin, "joyful, pretty") (**Figure 1** and **2**).

This phenotype is interesting commercially not only for having more tepals, but also for not producing pollen, since in mature stages of wild-type flowers it speckles tepals, clothes and the furniture. Many consumers remove the lily anthers prior to display, in order to avoid pollen flecking. Phenotypes such as *festiva* are, therefore, of great interest to breeding programs, and unravelling the molecular mechanisms behind this phenotype may lead to the development of molecular tools that facilitate replacing stamens by tepals.

Unfortunately, we were not able to perform molecular analysis of the *festiva* genotype in the time frame established to generate this thesis, but instead, we tried here to draw some hypotheses that can be explored later, raising some fascinating perspectives for further investigation.

Hypotheses regarding the *festiva* phenotype

The phenotype shown by *festiva* can, with its perfect homeotic changes of stamens into tepals, be easily pictured as an extension of an A-type function towards the third whorl, to the detriment of the C-type function in this whorl.

The challenge, however, is in understanding the exact mechanism through which this function replacement originated. We speculate that the whorl-specific homeotic change seen in *festiva* can be due to modifications of regulatory elements of A or C genes or to a third whorl-specific factor acting in stamen development.

Regulatory elements of transcription and the ABCDE model for flower development.

Transcription of developmental genes are under the regulation of *trans* and *cis*-regulatory elements that dictate the exact moment the genes are turned on and off in order to trigger a proper and regular developmental pattern for each species.

Trans-acting elements are protein factors operating in gene promoter regions (i.e. *cis*-regulatory regions) in order to be associated with other factors in the RNA polymerase complex and activate transcription or, alternatively, to inhibit the transcription of a given gene. Transcription factors, such as the MADS proteins, act as *trans*-acting elements in eukaryotic organisms.

Cis-regulatory regions are boxes present in gene promoters and other regulatory regions that modulate the spatial and temporal transcription pattern of a given gene by allowing selective interaction with transcription factors (i.e. *trans*-acting elements) and RNA polymerase. The CArG box is a known *cis*-regulatory element present in promoters of genes that interact with the MADS domain in order to assist their transcription (Pollock and Treisman, 1991).

The ABCDE model is mostly composed of MADS-box transcription factors ruling as key elements to activate (or repress) specific genes in flower development that will ultimately result in formation of floral organs. Changes in the sequence of their *cis*-regulatory regions or *trans*-acting elements may result in genetic misfunction, leading to homeotic changes in the flower pattern.

Lily flowers with their homeotic identity partially lost in the third whorl, as shown in **Figure 1b**, can be easily found. This change can be triggered by environmental conditions, suggesting a form of epigenetic regulation of homeotic genes involved in flower organ development. In the *festiva* phenotype, however, the homeotic change of the third whorl organs is regular and complete, suggesting a more severe cause than those triggered by stress conditions.

In this way, if a specific element regulating the expression of a C functional gene is affected, in *cis* or in *trans*, its transcription may be suppressed specifically in the third whorl and not in the fourth whorl, which would lead to specific homeotic changes of stamens into petals (or tepals, in the case of Liliaceae). Keeping in mind that A and C genes have antagonistic functions, alternatively, an extension of A-type gene domain that allows its transcription in the third whorl would repress the C function and raise specific homeotic changes of stamens in petals.

Important *cis*-regulatory regions of *AG* that control its spatial expression pattern were found in the second large intron of the gene (Sieburth and Meyerowitz,

1997). Additionally, regions responsible for independent *AG* activation in whorls two and three were identified intragenically (Deyholos and Sieburth, 2000). The zinc-finger transcription factor *SUPERMAN* also has its expression whorl-regulated by *cis* regions in the transcribed portion of the gene (Ito et al., 2003).

Trans-acting elements of the *AG* gene include positive and negative regulators of its expression, such as *LEAFY* (*LFY*; Weigel et al., 1992), *APETALA1* (*AP1*; Weigel and Meyerowitz, 1993) and *WUSCHEL* (*WUS*; Lenhard et al., 2001) as positive regulators and *LEUNIG* (*LUG*; Liu and Meyerowitz, 1995; Conner and Liu, 2000), *SEUSS* (*SEU*; Franks et al., 2002), *APETALA2* (*AP2*; Drews et al., 1991) and *STERILE APETALA* (*SAP*; Byzova et al., 1999) as negative regulators.

Both hypotheses, repression of C function or activation of A function, account for early stages of development, since it is highly plausible, by the phenotype given, that the A function is active in the third whorl and the C function is absent. *In situ* hybridisation of *festiva* floral buds in early developmental stages may answer which function is (firstly) mistranscribed in order to concentrate investigation on its genetic regulation.

A factor specific for the third whorl in the lily ABCDE model?

The development of sexual structures located in flower and the emergence of the flowering plant species is a key event in life evolution on the Earth.

Given the adaptive advantages flowers provided to plant species, it is not a surprise that the functions belonging to the ABCDE model are highly conserved in the many flowering species. Similarly, as the flowers present singular and conserved characteristics, one would expect to find evolutionary conservation also at the molecular level.

Lily species show a slightly different molecular mechanism to promote floral organ development when compared to other plant families, however, the fundamental features of the model seem to remain unchanged throughout evolution. The novel *festiva* phenotype has never been reported before in *Arabidopsis* or other species and, for that, it is conceivable that some aspect of the ABCDE may have changed since the last common ancestor of lilies and mustards.

Despite the overlapping nature of the ABC gene function found in *Arabidopsis* and *Antirrhinum* that gave rise to the early ABC model for flower development, it has been found that some species presented floral homeotic genes functioning in only one whorl. The petunia *green petal* (*gp*) mutant, which has a mutation in the B-type gene

pMADS1, shows the conversion of petals into sepals, but keeps the stamen identity in the third whorl (van der Krol et al., 1993; Tsuchimoto et al., 2000).

It has also been found that in maize different genes determine the C function in single whorls. The *AG* homologue *ZAG1* determines specifically the carpel formation, since *zag1* mutants show aberrant pistils and loss of floral determinacy but normal tassels with fertile pollen. The other maize *AG* homologue, *ZMM2*, is preferentially accumulated in stamens. These genes have overlapping but non-identical activities (Mena et al., 1996).

It is possible that the development of stamens in lily is also orchestrated by a homeotic factor exclusively found in the third whorl. If this is true, its lack of function would produce a phenotype that adopts the identity of an organ in a neighbour whorl. In the *festiva* case, stamens assumed a second whorl identity, indicating the conquest of the third domain by the A function.

Conclusions

The possibilities we raised are hypotheses that need further proof based on experimental results.

Nevertheless, all the hypotheses formulated here with respect to these floral phenotypes and the complexity involved in each of the hypotheses can give a glimpse on the sophistication of flower developmental pathways, the amount of information the field has gathered in the last 15 years and the amount of riddles scientists still have to solve in order to unravel fully the mystery of flower formation.

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CHAPTER SEVEN

The potential of Virus-Induced Gene Silencing for speeding up functional characterisation of transcription factors

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Abstract

Virus-induced gene silencing (VIGS) system has shown to be of great potential in plant reverse genetics. Advantages of VIGS over other approaches, such as T-DNA or transposon tagging, include the circumvention of plant transformation, methodological simplicity and robustness, and speedy results. These features enable VIGS as an alternative instrument in functional genomics, even in a high throughput fashion. The system is already well established in *Nicotiana benthamiana*, but efforts are being addressed to improve VIGS in other species, including monocots. Current research is focussed on unravelling the silencing mechanisms of post-transcriptional gene silencing (PTGS) and VIGS, as well as finding novel viral vectors in order to broaden the host species spectrum. Here, we discuss the advantages of using VIGS to assess gene functions in plants. We address the molecular mechanisms involved in the process, present the available methodological elements such as vectors and inoculation procedures, and we will show examples in which the system was applied successfully to characterize gene function in plants. Moreover, we analyse the potential application of VIGS in assessing genetic function of floral transcription factors from monocots.

Most used abbreviations

Antisense RNA (asRNA); **double-stranded RNA** (dsRNA); **hairpin RNA** (hpRNA); **micro RNA** (miRNA); **nucleotides** (nt); **post-transcriptional gene silencing** (PTGS); **RNA-directed RNA polymerase** (RdRP); **RNA-induced silencing complex** (RISC); **RNA interference** (RNAi); **small interfering RNA** (siRNA); **small RNA** (smRNA); **single-stranded RNA** (ssRNA); **small temporal RNA** (stRNA); **satellite virus-induced silencing system** (SVISS); **transcriptional gene silencing** (TGS); **virus-induced gene silencing** (VIGS).

Introduction

Reverse genetics is the search for gene functionality starting from a gene sequence. Knocking out genes is the strategy of reverse genetics most frequently used to infer about their functions. In *Arabidopsis*, two insertional mutagenesis approaches for gene disruption are predominant: transferred DNA (T-DNA) (Krysan et al., 1999) and transposon tagging (Parinov et al., 1999; Speulman et al., 1999). Although these approaches are powerful in providing novel mutants to the scientific community, they present some limitations, such as the impossibility of studying the function of duplicated genes, the difficulty to reach genome saturation, and the multiple insertional nature of these approaches, which frequently leads to concomitant disruption of several genes.

The use of tissue-specific promoters with antisense RNA (asRNA) and cosuppression technologies may prevent embryonic developmental obstacles but they are also labour intensive, time-consuming and, in some cases, unpredictable.

These constraints can be circumvented by new post-transcriptional gene silencing (PTGS) tools in which genes are silenced in a specific and efficient manner using less intensive and time-consuming technologies than the conventional ones (Wang and Wagner, 2003).

PTGS (Al-Kaff et al., 1998; Covey et al., 1997; Ratcliff et al., 1997) can be induced in plants by viral vectors harbouring specific genes using the virus-induced gene silencing (VIGS) system (Baulcombe, 1999; Kumagai et al., 1995), by inverted repeat transgenes producing hairpin transcripts (hairpin RNAs, hpRNAs) (Smith et al., 2000), by antisense RNA (asRNA) technology (Rothstein et al., 1987) or by gene overexpression (cosuppression) (Napoli et al., 1990; van der Krol et al., 1990). The VIGS system can be very helpful to assess gene function especially in species recalcitrant for transformation.

Here, we present an overview of the advantages and the current knowledge on genetics and biochemistry of PTGS and VIGS. In addition, we refer to the basis of molecular flower development in order to explore the possibility of using VIGS and model species for probing flower gene functions from heterologous species, such as *Lilium longiflorum*, which is our ultimate goal.

Advantages and Applications of Gene Silencing in Plants

Since the great majority of plant virus genomes consist of single-stranded RNA (ssRNA), gene delivery in the VIGS system is often performed with the inoculation of

viral vector transcripts synthesised *in vitro*. In the case of DNA viruses, such as geminivirus, inoculation is simplified because it requires only the corresponding viral DNA. Alternatively, the virus genome can also be inserted as a cDNA fragment into a binary vector to be introduced into the plant cell via agroinfection, facilitating the delivery process.

Genes involved in early developmental stages usually cannot be ascertained by gene disruption approaches, such as T-DNA and transposon tagging, because their lack of function leads to plant death. Using the VIGS system, these genes can be silenced after the critical point of germination, to elicit specific silencing from this moment on in order to understand their post-embryonic developmental roles (Peele et al., 2001).

In the genomics age, molecular biologists are looking for new alternatives to study gene function on a genome-wide scale. The high throughput techniques for gene discovery and expression analysis, such as whole genome sequencing and micro-arrays, demand efficient procedures to unravel gene functions in order to make them useful for fundamental and applied purposes. The information generated by those technologies can be combined with PTGS approaches to probe gene function in a high throughput fashion. This is already a reality in developmental genetics of the nematode *Caenorhabditis elegans* (Maeda et al., 2001).

Traditional gene knockout techniques use transformation as a delivery system and usually require tissue culture steps to regenerate silenced mutants. PTGS, and especially the VIGS system, can potentially be used as an important tool in the reverse genetic analysis to ease and speed up analysis of gene function without the requirement of time-consuming transformation and tissue culture procedures, since it can be applied in *ex vitro* developing plants, switching off genes specifically and in an efficient way, and allowing rapid monitoring.

VIGS is a promising technique also to analyse a high number of genes using only partial cDNA fragments instead of their full length coding regions as required for other procedures.

Scientists of genes involved in plant defence against pathogens and in cell development, such as transcription factors, are now challenging the VIGS system to facilitate functional analysis of plant genes. Large scale loss-of-function studies can now be performed using specific vectors that use highly efficient cloning systems, such as the Gateway recombination-based system, to insert gene fragments in a more efficient way (Liu et al., 2002a). The resulting phenotypes can be evaluated within days after inoculation instead of months, or even years, when the traditional methods that require transformation procedures are used.

It is important, however, to take into consideration that this system is efficient only for a few plant species until now, such as *Nicotiana benthamiana*, tomato and barley. Nevertheless, considerable efforts are being put into novel vectors in order to increase the number of species that responds efficiently to VIGS.

Summing up, the VIGS system can be used in reverse genetics to ease and speed up functional analysis without gene disruption, without requiring plant transformation, with only a fragment of the coding region, and allowing the study of multiple genes concomitantly.

Brief history of gene silencing

Post-transcriptional gene silencing (PTGS) is a general term that encompasses similar events occurring in diverse biological research fields. Plant biologists call it PTGS and cosuppression whereas RNA interference (RNAi) is the term originally used by those working with *C. elegans* and *Drosophila*, and quelling is used by fungi scientists (Cogoni and Macino, 2000). Since these phenomena show very similar mechanisms, RNAi is being used more recently as the general term to describe the event also in other kingdoms.

asRNA technology was discovered in plant research in 1987, with the inhibition of a nopaline synthase gene in tobacco cells given by the expression of its corresponding asRNA (Rothstein et al., 1987).

The first reports on gene silencing induced by cosuppression were produced in 1990 (Napoli et al., 1990; van der Krol et al., 1990) when scientists were trying to increase the purple pigmentation of petunia petals by sense overexpression of the *CHALCONE SYNTHASE* (*CHS*), a gene related to the anthocyanin pathway. This approach, however, induced variegated and white petals, caused by silencing of the endogenous chalcone synthase gene. Interestingly, the white colour of the petals was observed in following generations and, occasionally, some plants reverted to purple, indicating the event did not involve permanent DNA modification. This phenomenon was then designated cosuppression and intrigued the scientific community for a decade.

Similar events could be observed in the fungus *Neurospora crassa* when Cogoni et al. (1994) attempted to increase its orange pigmentation by introducing a gene involved in the carotenoid production. Instead of turning deep orange, the fungus bleached out in some cases. They denominated this process quelling. The bleached fungus frequently reverted to normal colour, indicating that the quelling process did not alter the DNA permanently either.

Guo and Kemphues, in 1995, showed the first evidence that sense RNA could instigate gene silencing in *C. elegans*. Intending to use asRNA to silence a gene that regulates embryo symmetry, they also observed gene silencing when using the sense RNA as control.

In 1998, Fire and colleagues, also working with *C. elegans*, found that although single-stranded asRNA could trigger gene silencing, dsRNA was much more effective. They coined the term RNA interference (RNAi) for this process.

The VIGS system was established from studies on the sequence homology between a virus and either a transgene or an endogenous gene that would cause PTGS (Kumagai et al., 1995; Lindbo et al., 1993). In this system, a virus vector carrying a copy of the gene to be silenced is introduced into the cell, the cellular machinery recognises the viral threat and releases a protective defence to destroy not only viral genes but also any extra gene being carried by the viral vector, implicating also any native or transgenic homologous transcripts of the infected cell (Ruiz et al., 1998; Waterhouse et al., 2001).

The first cues on the molecular mechanism underlying PTGS were derived from studies in plants, with a class of small RNAs of about 25 nt identified as the triggering signal for gene silencing (Hamilton and Baulcombe, 1999). Genetic and molecular studies have confirmed that cosuppression, RNAi, quelling and VIGS share similar mechanisms of gene silencing that is widely present in eukaryotic species (Gura, 2000).

Current mechanism of PTGS in plants

Characterization of the PTGS mechanism is still in its infancy. Much effort is being allocated to elucidate the genes involved in the defensive response for gene silencing and its biochemistry. The mechanisms seem to be conserved throughout evolution given that homologous genes involved in the process were already found in species of fungi, animals and plants.

The present model for gene silencing includes three phases: initiation, maintenance, and signal amplification and spreading (Nishikura, 2001). **Figure 1** illustrates the mechanism of gene silencing. dsRNA is the triggering factor for gene silencing and the first phase involves its synthesis or formation, recognition, and the production of small interfering RNA (siRNAs) fragments. dsRNAs can be generated by the RNA virus replication mechanism, which includes the formation of dsRNA by an RNA-dependent RNA Polymerase (RdRP), by hpRNA, originated from a bidirectionally cloned transgene or by an asRNA cloning strategy. Still in the initiation

phase, the next step involves mRNA targeting and degradation by the enzyme *Dicer* (Bernstein et al., 2001) that recognises and cleaves dsRNAs from both ends into siRNAs of 21 to 23 nt (Zamore et al., 2000).

These siRNAs, alternatively referred to as guide RNAs, are identification sequences for the RNA-induced silencing complex (RISC), a protein-RNA effector nuclease formation of about 500 kDa, in the maintenance phase (Hammond et al., 2000). RISC presents exo- and endonuclease activities, an RNA homology-searching activity and a helicase to unwind the dsRNA. This complex can be activated by unwinding siRNAs in order to use their single-stranded siRNA sequences for identification and degradation of complementary transcripts (Nykänen et al., 2001).

The third phase concerns the amplification of a silencing signal and its spreading throughout the target transcript. This signal is being identified as the siRNAs originated in the preceding phases, and it is alternatively referred to as transitive RNAi. Guided by an RdRP in order to amplify and potentiate the silencing response, siRNAs act as activators (primers) for dsRNA polymerization using ssRNA as a template. In plants, siRNA can induce RNA polymerisation in both 3'→5' and 5'→3' directions whereas in animals RdRP travels only in one direction (3'→5') along a certain mRNA to amplify the silencing signal (Vaistij et al., 2002). Production of dsRNA feeds the initiation phase for production of more siRNAs and the process continues in a reiterated fashion.

Grafting experiments have proven that systemic dispersion of a silencing signal does happen in plants, being called systemic acquired silencing (SAS). An elegant experiment showed *beta-glucuronidase* (*GUS*) silencing in a *GUS*-expressing scion grafted onto a *GUS*-silenced rootstock. The signal moved unidirectionally from source to sink organs, getting across up to a 30-cm wild-type stem grafted between the *GUS*-expressing scion and the *GUS*-silenced rootstock (Palauqui et al., 1997). Despite systemic dispersion has been proven to occur, the nature of this mobile silencing signal remains elusive. Its specificity indicates, however, that it may consist at least partially of a nucleic acid.

Imperfect complementarity between target transcripts and small RNAs (smRNAs) can also trigger gene silencing. These ~22-nucleotide (nt) smRNAs with imperfect base pairing belong to a class called micro RNAs (miRNAs) (previously termed small temporal RNAs, stRNAs). They are also produced by *Dicer* in the initiation step, and thought to be involved in translation repression (Jones, 2002) and in controlling developmental pathways in plants (Baulcombe, 2002; Cerutti, 2003; Hunter and Poethig, 2003; Reinhart et al., 2002), acting in specific mRNA destruction by RISC (Tang et al., 2003).

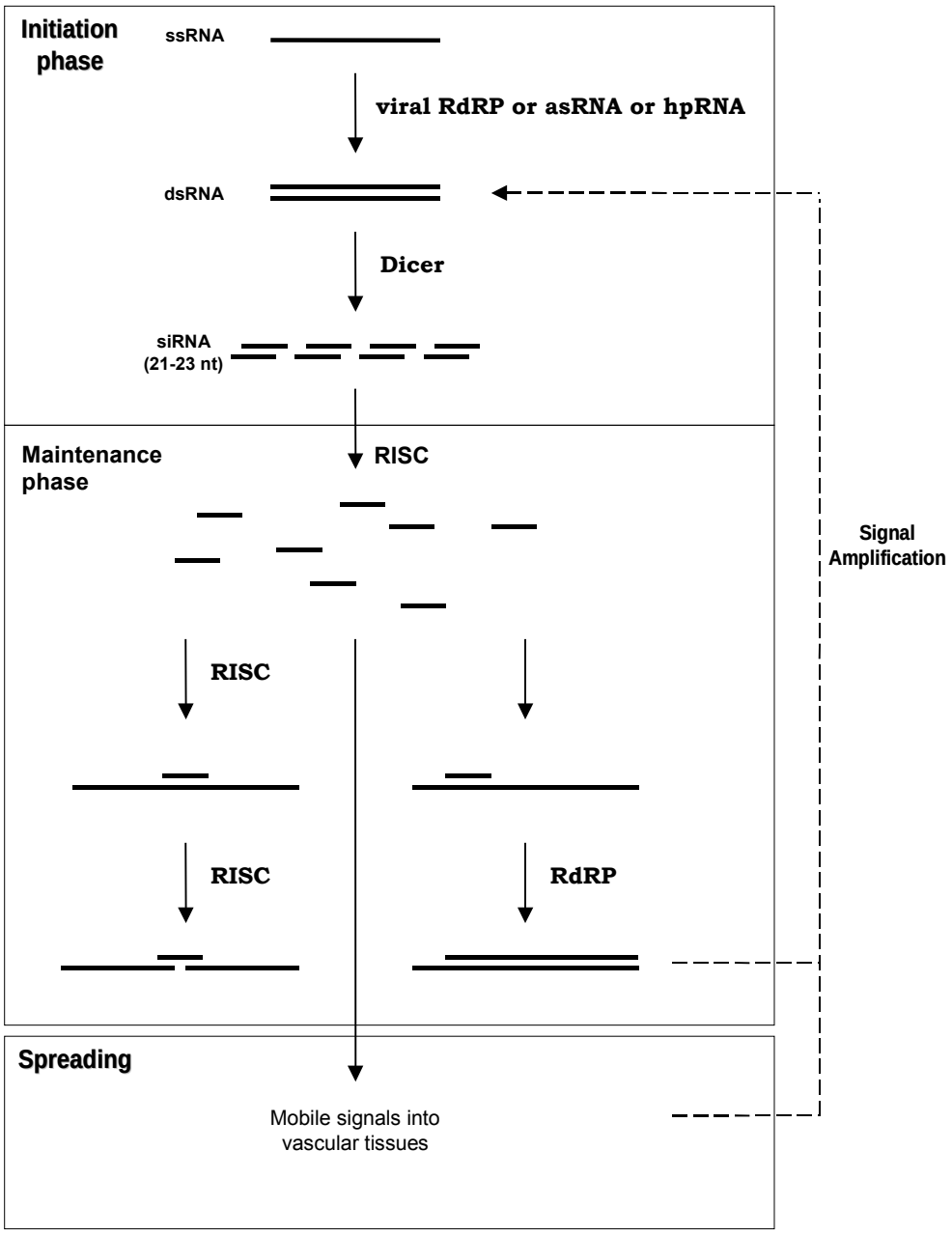

Figure 1. Current model of post-transcriptional gene silencing. The initiation phase can be triggered by a viral RdRP that makes a complementary RNA strand using the single-stranded as a template, by a transgenic antisense RNA or by a hairpin RNA. Double stranded RNA is recognized by the Dicer enzyme and is chopped into pieces of 21-23 nt called siRNAs. In the maintenance phase, RISC unwinds the siRNAs, triggers the surveillance mechanism to find siRNA complementary RNA in the cell, and inactivates them with its RNase activity. Alternatively, siRNAs can serve as primers for an RdRP to make dsRNA during the silencing signal amplification, in a feedback fashion. Moreover, siRNAs is believed to constitute (at least partially) mobile signals that spread the gene silencing mechanism in other parts of the plants through vascular tissues.

Table 1. Genes involved in post-transcriptional gene silencing.

Genes in Arabidopsis	Orthologues	Protein Domains	Functions	Mutant phenotype	References
AGO1	*Neurospora QDE2* *Caenorhabditis RDE-1* *Drosophila AGO1*	PAZ PIWI (PPD)	Protein-protein interactions ?	Changed leaf morphology Less secondary meristems Altered plant architecture Sterility Impaired PTGS	Bohmert et al., 1998 Fagard et al., 2000 Hammond et al., 2001
AGO4		PPD	Protein-protein interactions	Suppression of SUP silencing	Zilberman et al., 2003
DCL1 (*SIN/ SUS/ EMB76/ CAF*)	*Drosophila Dicer* *Caenorhabditis DCR1* Rice *DCL*	PAZ RNase III RNA helicase	Protein-protein interactions Dicer RNA unwinding	Developmental abnormalities (ovules, embryos, floral meristems) Reduced miRNA accumulation	Finnegan et al., 2003 Golden et al., 2002 Park et al., 2002 Schauer et al., 2002
HEN1	Putative carboxy-terminal homology with sequences from human, mouse, fungi, *Caenorhabditis*, *Drosophila*. Extensive sequence homology with rice	novel	?	Dwarfism Changed leaf morphology Late flowering Floral organ defects AxC floral function interactions Reduced miRNA accumulation	Chen et al., 2002 Golden et al., 2002 Park et al., 2002
SDE1/SGS2	Tomato *RdRP* *Neurospora QDE1* *Caenorhabditis EGO-1*	RdRP	RNA polymerase	Defective leaf development Enhanced viral susceptibility Impaired PTGS	Dalmay et al., 2000 Mourrain et al., 2000
SDE2		?		Same as *SDE1/SGS2*	Hamilton et al., 2002
SDE3	*Drosophila dmgb110* Mouse *mmgb110*	RNA helicase	RNA unwinding	Impaired PTGS	Dalmay et al., 2001
SDE4		?		Suppression of DNA methylation Suppression of systemic silencing	Hamilton et al., 2002 Zilberman et al., 2003
SGS1		?		Impaired PTGS	Elmayan et al., 1998
SGS3	Rice gene *X*	XS-coiled-coil zf-XS	Protein-protein interactions Enzymatic activity (?)	Same as *SDE1/SGS2*	Mourrain et al., 2000 Bateman, 2002

The differences between miRNA and siRNA were emphasised by Bartel and Bartel (2003), that generally termed all the small-sized RNAs found in the cell as tiny RNAs. In general terms, miRNAs are transcribed directly from the genome by their own encoding genes, show high nucleotide identity within related organisms, and are used by the cell to regulate different genes. On the other hand, siRNAs are originated from viruses, mRNAs, transposons or heterochromatic DNA, with higher nucleotide divergence in related species and used to silence nearly identical genes from which they are originated. A great question still to be addressed is whether the same type of RISC can be programmed by miRNAs and siRNAs indistinctly or not.

PTGS in plants is not solely triggered by dsRNA directly formed from expressed hpRNA, asRNA or virus expression but it is also elicited by high expression of sense transcripts from a transgene. This phenomenon is called cosuppression and is thought to be correlated with the accumulation of free 3' hydroxyl termini that are not covered by poly(A)-binding proteins, leading to formation of hairpin structures that would instigate the silencing process by the aforementioned mechanisms (Hammond et al., 2001; Lipardi et al., 2001).

Genes involved in the plant gene silencing mechanism

Mutations in genes involved in the gene silencing pathway can be found in individuals that fail to have their genes silenced by PTGS. Interestingly, these mutants are frequently associated with developmental aberrations.

Although a full comprehension of the PTGS genetic network is still waiting to be revealed, it is clear that PTGS and VIGS pathways share common players, which may also be important elements in normal plant development in the recently disclosed RNA metabolism.

In the following, we present an updated set of genes that are involved in gene silencing mechanisms, the main features of the gene products, and the phenotypes produced by their mutant alleles in plants. **Table 1** brings an overview of the genes involved in PTGS.

The gene coding for the enzyme *Dicer-1* (*DCR1*) from *Drosophila*, which is active in the dsRNA cleavage process, is related to the gene coding RNase III from *Escherichia coli*. In *Arabidopsis*, three presumed genes related to *DCR1* (*SHORT INTEGUMENTS1*, *SIN1*; *SUSPENSOR1*, *SUS1*, named previously *EMBRYO DEFECTIVE76*, *EMB76*; and *CARPEL FACTORY*, *CAF*) were found to be mutant alleles with different strengths of the same gene, and renamed *DICER-LIKE1* (*DCL1*) (Golden et al., 2002; Schauer et al., 2002). All these *DCL1* mutants showed developmental abnormalities. *CAF* loss-of-function causes overproliferation of the floral meristem (Jacobsen et al., 1999) while *SUS1* and *SIN1* mutations are associated respectively with embryo abnormalities (Schwartz et al., 1994) and ovule defects (Robinson-Beers et al., 1992). *Dicer* homologues contain a PAZ domain (Cerutti et al., 2000), which is thought to mediate interactions in the RISC. *Dicer* homologues also bear RNase III motifs and an RNA helicase domain (Hammond et al., 2001).

Four *Dicer* homologues have been found in the *Arabidopsis* genome and the allelic series of *DCL1* encompasses already ten mutant members (Finnegan et al., 2003; Golden et al., 2002). In the rice genome, two *Dicer* homologues have been identified, corroborating with the assumption that the silencing mechanism is also conserved in monocots (Golden et al., 2002).

HUA ENHANCER1 (*HEN1*) was discovered recently in *Arabidopsis* as a new player in RNA metabolism, being required, together with the *Dicer* homologue *DCL1*, for the accumulation of miRNAs (Park et al., 2002). *HEN1* is associated with growth of stems, leaves and floral organs, with male and female fertility, with floral meristem identity, and with floral determinacy. *HEN1* seems to act antagonising the A functional genes and promoting the C function (Chen et al, 2002). Interestingly, a 18-21 nt RNA

transcript (called *MIR172*) was identified in inflorescences, leaves and stems of wild-type *Arabidopsis* and showed sequence complementarity to *APETALA2* (*AP2*), whereas it was hardly detected in *hen1* and *dcl* mutants (Park et al., 2002). There is evidence that miRNA metabolism and PTGS are intrinsically related phenomena, since *dcl1* and *hen1 Arabidopsis* mutants showed drastic reduction of miRNA levels, and similar developmental aberrations (Park et al., 2002).

A gene called *SILENCING DEFECTIVE1* and *SUPRESSOR OF GENE SILENCING2* (*SDE1*/*SGS2*) (Dalmay et al., 2000; Mourrain et al., 2000) was identified in PTGS-defective *Arabidopsis* lines and codes for an RdRP. In plants, RdRP was first characterized in tomato (Schiebel et al., 1998). The role of this gene is proposed to synthesise a dsRNA PTGS initiator. When instigated to silence transgenes, *sde1*/*sgs2* mutants showed low levels of correlated tiny RNAs, indicating the gene is important for transgene PTGS (Dalmay et al., 2000). A virus-induced PTGS was enhanced in these mutants instead, since viruses provide their own RdRP gene in order to produce dsRNA and provoke gene silencing. This result strongly indicates that PTGS is indeed an antiviral defense mechanism to trigger degradation of RNA derived from exogenous DNA (Mourrain et al., 2000).

Together with *SGS2, SGS1* was identified in *Arabidopsis* mutants that failed to promote PTGS (Elmayan et al., 1998), indicating that the product from both genes are *trans*-acting elements involved specifically in PTGS.

SGS3 is another gene related to the PTGS in *Arabidopsis*. Its lack of function shows the same phenotype reported above for *SGS2* (Mourrain et al., 2000). However, in contrast to *SGS2*, the *SGS3* product is not related to any functionally characterised protein. Recently, SGS3 was found to share sequence homology to the rice gene *X*, with two conserved domains: an XS domain that indicates a putative enzymatic activity conjugated to coiled-coil structure, suggesting an oligomerisation site; and a zf-XS domain with a yet elusive function (Bateman, 2002).

SDE3 was found in *Arabidopsis* as a gene encoding an RNA helicase required for the PTGS pathway (Dalmay et al., 2001). *sde3* mutant showed the same phenotype described for *sde1*/*sgs2*, but its function in the proposed mechanism requires further analysis. Likewise, the *SDE2* loss-of-function mutant also shows similar phenotype and its function is still uncharacterised. *SDE4*, however, shows a distinct phenotype and may be associated with a step required for DNA methylation (Hamilton et al., 2002).

In *Arabidopsis*, the *ARGONAUTE1* (*AGO1*) gene was identified in mutants producing altered leaf morphology, reduced number of secondary meristems, and

sterility due to flower developmental defects (Bohmert et al., 1998). *AGO1* belongs to a gene family present in many kingdoms that counts at least eight members in the *Arabidopsis* genome (Fagard et al., 2000; Hammond et al., 2001). Two main domains are found in *AGO1*: the PAZ domain that may be implicated in protein-protein interactions, and the PIWI domain, with an unidentified function. Both domains are referred to as the PPD domain.

Mutations in other genes of this family induced similar ontogenic defects and PTGS impairment as observed in *ago1* mutants. Members of the *AGO* gene family have also been linked to histone modifications and transcriptional gene silencing in several eukaryote species (Hall et al., 2002; Mochizuki et al., 2002; Pal-Bhadra et al., 2002; Taverna et al., 2002; Volpe et al., 2002). In plants, mutation of *AGO4* led to suppression of silencing of *SUPERMAN* (*SUP*), a zinc-finger homeotic transcription factor, through its *clark kent* (*clk*) epigenetic alleles, rescuing the wild-type phenotype in *Arabidopsis*. *AGO4* appears to be mechanistically related to *SDE4* and it is plausible that both are involved in generating long siRNAs specialised in the chromatin level of gene silencing (Zilberman et al., 2003) rather than being implicated in VIGS.

It is now clear that PTGS is spread in the diverse kingdoms, sharing conserved factors in species such as *Arabidopsis* and *Drosophila*. The challenge now lies in understanding the genetic regulation of gene silencing pathways in order to establish the network interactions.

Vectors for Virus-induced Gene Silencing

The regions of the plant that can be affected by gene silencing depend mostly upon the viral vector characteristics. Silencing vectors usually travel systemically through phloem in the vascular tissues to most parts of the plants. Although most of viruses do not penetrate meristems, some have been demonstrated to deliver the silencing signal to meristematic regions of the plant (Peele et al., 2001).

Important characteristics of a viral silencing vector are its effectiveness to induce silencing; its capability of infecting and inducing silencing in growing regions of the plant; its genome size (due to cloning steps for fragment insertion, since usually the smaller the vector the easier the cloning); its genome partition; the type of nucleic acid its genome is composed of (given that RNA plant viruses are the most common but DNA viruses can facilitate the inoculation for allowing the use of DNA instead of the unstable RNA); its host range; whether its genome is or can be made available in binary vectors (to facilitate inoculation via *Agrobacterium*); and its safety for individuals and the environment.

Tobacco mosaic virus (*TMV*) was the first viral vector used to successfully elicit VIGS of an endogenous gene in a plant species (Kumagai et al., 1995). This work is seen as a breakthrough in the plant PTGS research field.

Potato virus X (*PVX*) (Ruiz et al., 1998) followed as the next viral vector used to carry a gene into a plant cell and trigger silencing via VIGS. Although effective, its incapability of infecting meristems is a great disadvantage, since delivering silencing to meristematic tissues is a *sine qua non* for studying genetic functions involved in developmental pathways.

The advent of the *Tobacco rattle virus* (*TRV*) vector for VIGS (Ratcliff et al., 2001) opened the possibility of studying genes expressed in early organ development because of its ability to reach growing points and to deliver the silencing signal to meristems. The cloning of its genome into binary *Agrobacterium tumefaciens* plasmids facilitated immensely the infection process.

TRV is a ssRNA virus with a bipartite genome. The component called RNA1 encodes, among other genes, an RdRP whereas its genome partner, called RNA2, encodes the coat protein (Angenent et al., 1986; MacFarlane, 1999). The target gene fragment for silencing is inserted into the RNA2 element. Inoculation, either mechanical or via agroinfiltration, requires the presence of both genome components. In the case of agroinfiltration, two different *Agrobacterium* clones, one carrying the RNA1 genome and another with the RNA2 containing the target gene fragment, are mixed together and co-infiltrated into the leaf tissues (English et al., 1997).

Notwithstanding the advantages, such as facility of application with leaf agroinfiltration, prompt results within days after inoculation, and independence on the whole coding sequence of the target gene to elicit silencing, in the case of high throughput goals, there are additional requirements to be met, including a high cloning efficiency, in order to enable a high number of candidate sequences to be placed into the vector within a short period. This condition is fulfilled by a *TRV* vector based on the Gateway cloning technology (Invitrogen Life Technologies). No restriction enzymes are involved in the Gateway cloning steps but, instead, recombination sites that flank the gene fragment are target of recombinases which translocate very efficiently the fragment into the vector (Liu et al., 2002a).

A significant limitation of the VIGS system is that *TRV* and most of the other vectors available worked very well in *N. benthamiana* and tomato, but do not show efficacy in *Arabidopsis*, in which a myriad of genes are still looking forward to being functionally analysed. Additionally, a vector showing capability of infecting monocot species, such as maize, rice and lily, is also greatly desired. As a reference, **Figure 2**

shows the systematic classification of the most important plant species cited throughout the text.

Geminivirus vectors are very promising tools, especially because, due to their DNA-based genome, they enable the direct use of plasmid DNA infection instead requiring *in vitro* transcription for mechanical inoculation. The *Tomato golden mosaic geminivirus* (*TGMV*) vector (Kjemtrup et al., 1998) was effective in meristematic tissues of *N. benthamiana* for silencing the *PROLIFERATING CELL NUCLEAR ANTIGEN* (*PCNA*), an essential gene associated with DNA replication (Peele et al., 2001).

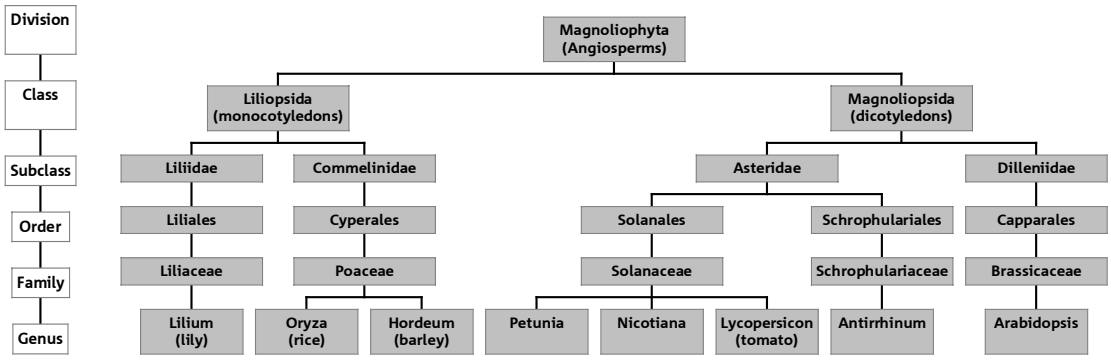

Figure 2. Systematics of species frequently cited in the text for reference. PTGS is being well established especially among Solanaceae members. Efficient VIGS approaches for other organisms, such as *Arabidopsis* and monocot species are being developed only recently.

The taxonomic information was retrieved (Aug/14/2003) from the *Integrated Taxonomic Information System* (*ITIS*) [http://www.it is.usda.gov].

Satellite viruses use the genome information contained in helper viruses in order to replicate and move inside the plant. A VIGS system based on the *Satellite tobacco mosaic virus* (*STMV*), which uses the *TMV* strain U2 as a helper, was reported recently (Gosselé et al., 2002). This method is called Satellite Virus-Induced Silencing System (SVISS) and shows some advantages over other systems, such as high stability and accumulation of the silencing signal in the infected tissues, easy cloning due to its small genome size, and attenuated symptoms of virus infection. Enzymes involved in several metabolic pathways were effectively silenced, showing clear phenotypic alterations four weeks after inoculation.

The *Cabbage leaf curl geminivirus* (*CbLCV*) has also a great potential as a VIGS vector since it has been shown to infect and trigger silencing of transgenes and endogenous genes in *Arabidopsis*. This vector reported here was the first to infect

effectively this species, which could mean a major breakthrough in the plant genomics field (Turnage et al., 2002).

The *Barley stripe mosaic virus* (*BSMV*) vector induced successfully gene silencing in barley (Holzberg et al., 2002) using *PDS* gene fragments from barley, rice and maize. *BSMV* has a broad host range in the Poaceae family, including rice, maize, wheat and oats (Brunt et al., 1996 onwards), which demonstrates the possibility of using this vector to address functional gene analysis via VIGS in monocot species, particularly interesting for those with high economic value and of great biological relevance as model species.

Inoculation procedures

The first approach designed to deliver the viral genome harbouring a fragment of the gene to be silenced was through direct cell inoculation with RNA transcripts, since the first vectors were RNA viruses. A vector containing the viral genome and the gene fragment of interest under a bacterial promoter (for example, SP6, T3 or T7) allows *in vitro* transcription of large amounts of RNA. Plants are dusted with an abrasive substance (such as carborundum), the RNA solution is applied to the leaves and friction is exerted in order to deliver the RNA into the parenchyma cells.

Geminiviruses as VIGS vectors present the advantage of eliminating the transcription step, requiring, as DNA viruses, just the direct inoculation of plasmid DNA. Since *in vitro* transcription on a wide scale can be economically prohibitive, plasmid DNA isolation provides a much cheaper alternative for working with a high number of vectors. Inoculation of plasmid DNA can be performed mechanically, as described above for RNA, or by microbombardment instead, in which tungsten or gold microprojectiles coated with the plasmid DNA are introduced in seedlings (Kjemtrup et al., 1998; Peele et al., 2001; Turnage et al., 2002).

Agroinfection uses *Agrobacterium* binary vectors to transfer the genetic information into plant cells. In the VIGS system, they are engineered to harbour the viral genome holding the gene of interest. The leaf agroinfiltration method delivers the viral genome into plant cells in order to infect and initiate the transient silencing process by injecting *Agrobacterium* culture into the leaf parenchyma with a needleless syringe. Besides its inoculation efficiency and ease, leaf agroinfiltration is routine only for a few species, especially from the Solanaceae family, such as *N. benthamiana* and tomato. Additional efforts must be undertaken to analyse if this technique can also be applied efficiently in other plant families as well.

In tomato, an alternative approach to the leaf agroinfiltration is spraying the bacterial culture with a paint airbrush on the leaves (Liu et al., 2002a). The authors induced *PHYTOENE DESATURASE* (*PDS*) silencing with a *TRV* vector cloned in *Agrobacterium*. While gene silencing was achieved in 50% of the plants using the agroinfiltration method, the airbrush approach resulted in 90 to 100% gene silencing.

Target genes and silencing fragments in PTGS and VIGS

The first gene reported to be silenced by PTGS was the *CHALCONE SYNTHASE* (*CHS*), in petunia, of which overexpression was expected to induce deep purple petals but it induced white or variegated flowers instead (Napoli et al., 1990). A similar event occurred with the first gene silencing report in fungi, known as quelling, with the overexpression of the genes involved in carotenoid metabolism causing albino phenotype, instead of showing the anticipated deeper orange color (Cogoni et al., 1994).

The *PDS* (Demmig-Adams and Adams, 1992) is an ideal gene to demonstrate the effectiveness of VIGS. *PDS* participates in the carotenoid metabolic pathway, acting in the antenna complex of the thylakoid membranes and protecting the chlorophyll from photooxidation. Its silencing results in a drastic decrease of carotene content in the leaves, leading to a photobleaching symptom that is easily detectable. It has being used frequently as a positive control of the system (Holzberg et al., 2002; Liu et al., 2002a; Ratcliff et al., 2001; Ruiz et al., 1998; Turnage et al., 2002). **Figure 3** shows the phenotypes of leaf bleaching in *N. benthamiana* triggered by *PDS* fragments from tomato and lily.

Vectors harbouring a *GREEN FLUORESCENT PROTEIN* (*GFP*) gene fragment have been used occasionally to demonstrate gene silencing in transgenic plants (Peele et al., 2001; Ratcliff et al., 2001; Ruiz et al., 1998; Turnage et al., 2002) and, together with *GUS*, they are considered the genes of choice for studies on suppression of gene silencing.

Functional characterization of a *CELLULOSE SYNTHASE A* (*CES A*) gene series from tobacco (*N. tabacum*) was undertaken in order to silence their orthologues in *N. benthamiana* using a VIGS vector based on *PVX* (Burton et al., 2000). Silencing of this function resulted in a dwarf phenotype, decrease of cellulose content in the cell wall, and increase of the homogalacturan levels.

Genes involved in resistance responses against pathogens have also been studied via VIGS. The tobacco *Rar1, EDS1* and *NPR1/NIM1*-like genes were proven

to be involved in the resistance to *Tobacco mosaic virus* (*TMV*) using a *TRV*-based vector in *N. benthamiana* (Liu et al., 2002b).

In *Arabidopsis*, *CONSTITUTIVE TRIPLE RESPONSE 1* and *2* (*CTR1* and *2*) encode protein kinases that negatively regulate ethylene responses. Their loss-of-function induces severe dwarfism and constitutive expression of ethylene-inducible genes. VIGS was applied in tomato to confirm the functional homology between *CTR1* and *2* from *Arabidopsis* and its homologue sequences (Liu et al., 2002a). Silencing of tomato *CTR1* and *2* homologues in this species was able to induce the same lack-of-function phenotype observed in *Arabidopsis ctr1* mutants.

Using cosuppression approaches, it was found that it is not necessary to use the whole coding region in order to induce gene silencing, but instead, only a fragment with enough homology to the target gene is already sufficient to elicit the silencing process (Ruiz et al., 1998). Conserved boxes of gene families can potentially trigger silencing of other members concomitantly (Ratcliff et al., 2001). One example is the crossed cosuppression of MADS box transcription factors family in petunia. Overexpression of the *PETUNIA FLOWERING GENE* (*PFG*) induced cosuppression of *PFG* itself and also of *FLOWERING BINDING PROTEIN 26* (*FBP26*), which share high overall sequence homology with *PFG (*74%), particularly within the MADS box (88%) (Immink et al., 1999). In this way, and in accordance with the molecular model for gene silencing, it is reasonable to think that the sequence homologies found in conserved boxes of gene families are more important for multiple gene silencing than their overall homologies. This may also be true for VIGS.

Gene fragments of around 300 to 800 nt have been frequently used in VIGS systems (Holzberg et al., 2002; Liu et al., 2002a; Ratcliff et al., 2001), although smaller fragments, even with 23 to 60 nt with sufficient homology may also be effective (Thomas et al., 2001). Gosselé and colleagues (2002), working with the SVISS system, reported that insert sizes larger than 300 nt led to lower levels of gene silencing, whereas fragments of about 100 nt induced silencing successfully.

Geminiviruses impose strict limitations to the size of DNA insertions they can carry. Fragments of 92 to 154 nt of the *SULFUR* (*SU*) gene were inserted in the vector and showed effectiveness in inducing specific gene silencing with *TGMV*. Additionally, 51 nt of a homologous sequence seemed to be near the lower limit to trigger gene silencing in this system, while gene fragments of 455 and 935 nt were less efficient in triggering gene silencing than the smaller fragments (Peele et al., 2001).

All these new possibilities and improvements of VIGS are now challenging the system for its efficiency in probing functions of important genes from diverse plant

species, in our case particularly of those linked to flower development, such as the MADS-box transcription factors.

Perspectives for reverse genetics of monocot genes based on VIGS

It has been demonstrated that the gene silencing mechanism is conserved and the phenomenon can happen effectively in monocots (Iyer et al., 2000), even though the VIGS system is not well established in these species yet.

The first experiment addressing the induction of transient gene silencing in a monocot species used microprojectile bombardment, a common transformation procedure for monocots, to deliver hpRNA into leaf epidermal cells of maize, wheat and barley (Schweizer et al., 2000). However, researchers have been noticing gene silencing in transgenic plants of several Graminae species already a long time ago, including rice, barley, maize, wheat and sugarcane, next to bulbous monocot species such as lily, orchids and others (reviewed by Iyer et al., 2000).

PTGS was firstly proven to occur in rice protoplasts expressing the beta-glucuronidase (*GUS*) gene (Kanno et al., 2000). Moreover, virus resistance in monocot species has been accredited to PTGS in some cases, such as in rice (Pinto et al., 1999), sugarcane (Ingelbrecht et al., 1999) and ryegrass (Xu et al., 2001).

Our preliminary results indicate that a *PDS* fragment derived from a monocot, such as lily, can elicit VIGS in *N. benthamiana*, in spite of the remote evolutionary relationship between the species (**Figure 3**). VIGS was induced by a TRV vector containing a 300-bp *PDS* fragment derived from lily. This lily fragment shows 70% identity to the *PDS* from *N. benthamiana*. Although the symptoms were not as strong as the bleaching elicited by the tomato *PDS* fragment, it shows that VIGS may work in a heterologous dicot system using a monocot gene fragment.

The first demonstration of VIGS in monocots was recently accomplished by Holzberg et al. (2002). Using the *Barley stripe mosaic virus* (*BSMV*) vector with a barley *PDS* gene fragment, they were able to induce silencing of the *PDS* gene in barley, rice and maize. *PDS* from *N. benthamiana* failed to provoke silencing in barley, though. This result can be attributed by the diverged nucleotide identity between the *PDS* genes from barley and *N. benthamiana*, which is 74%, whereas it is 91 and 89% between barley and the other two monocots that induced gene silencing, rice and maize, respectively.

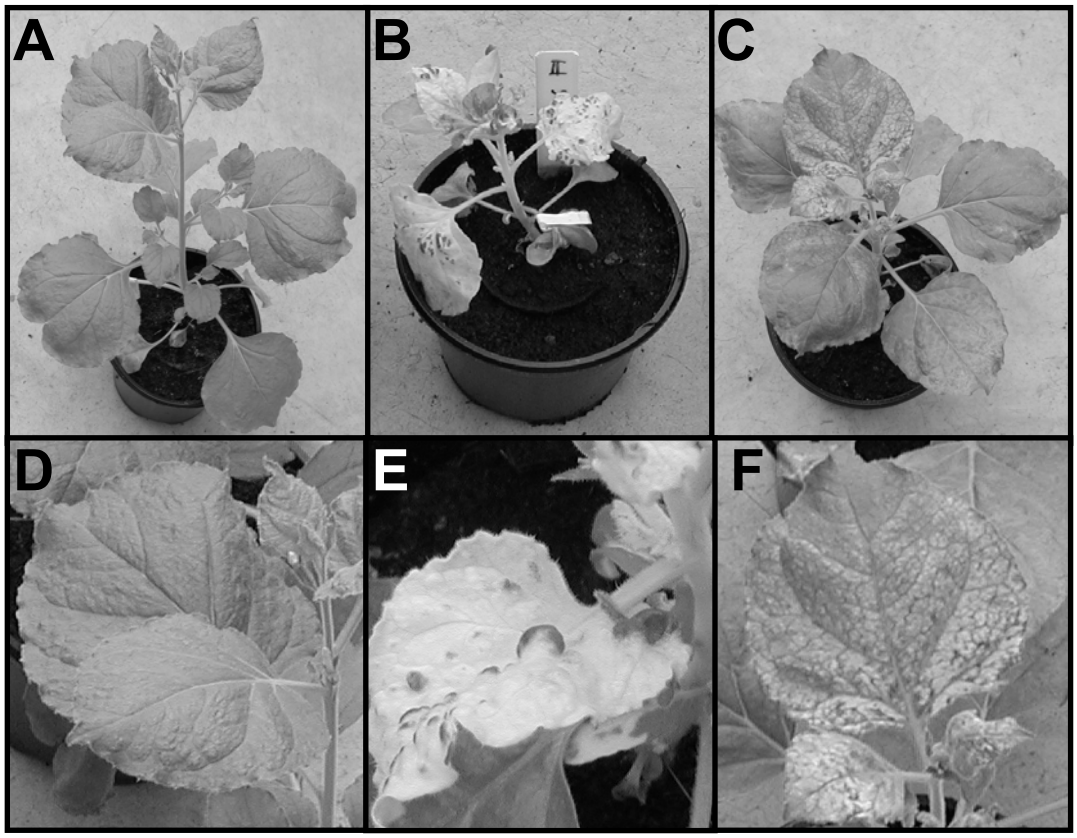

Figure 3. *Nicotiana benthamiana* at 22 days post inoculation (dpi). Plants were agro-infiltrated with TRV-derived vector carrying a *PDS* fragment from tomato (**B**, **E**) or lily (**C**, **F**) using agroinfiltration. Gene silencing can be observed by the leaf bleaching phenotype. Notice the strong silencing triggered by the *PDS* from tomato, whereas the lily *PDS* fragment induced a much weaker bleaching. Symptoms were already clear at 10 dpi. Mock plants are shown in **A** and **D**.

Besides the convincing results obtained with barley, it would be too early to establish this species as a model crop for VIGS in monocots, since rice would be the first option for this position given its importance as staple food worldwide and as an already established model organism in molecular biology, with its genome completely sequenced.

In fact, a novel VIGS vector is about to be released for rice (Ding and Nelson, 2003). The system is based on the *Brome mosaic virus* isolated from the grass *Festuca pratensis* (*F-BMV*). It is a tetrapartite virus that also infects barley, rice (both *japonica* and *indica*) and maize efficiently and was engineered to boost gene silencing in grasses. Additionally, this virus has no known insect vector nor is transmitted through seeds, increasing the safety of this system. Full reports are still being awaited but preliminary information already shows the potential of this vector for assessing

functions from monocot genes using closely related species as a heterologous system instead of making use of more diverged, dicot species.

These recent reports are providing good prospects for the possibilities of using VIGS for reliable and straightforward reverse genetics in monocot species, including the potential for easily and speedily studying gene function of flower homeotic genes from grasses and bulbous species.

In the following sections, we present an overview of the genetic regulation of flower development and propose using the VIGS system in functional characterization of flowering genes.

MADS-box transcription factors and flower development

MADS-box genes code for transcription factors involved in developmental pathways of eukaryotic organisms. In plants, they are implicated in diverse developmental programs, such as root development, fruit ripening, dehiscence zones, and especially, floral initiation and organ formation (Causier et al., 2002). The *Arabidopsis* genome encompasses 107 MADS-box genes (Parenicová et al., 2003), representing a significant portion of the developmental genes identified so far.

Floral initiation comprehends the process in which vegetative apical meristems develop into floral meristems. The complete picture is still to be drawn but an already complex network of genes involved in flower development pathways triggered by light, temperature, nutrients and hormones was delineated (Blázquez, 2000), showing several MADS box transcription factors playing fundamental functions in this process.

After floral initiation, transcription of homeotic genes is induced to form sepals, petals, stamens and carpels in the floral meristem. Studies with *Arabidopsis*, *Antirrhinum* and petunia led to the current ABCDE model for flower development in which many MADS-box homeotic transcription factors execute essential roles.

The ABCDE model explains the flower formation as the result of differential expression of homeotic genes, in which sepals, petals, stamens and carpels are formed by the respective expression of genes with A, A+B+E, B+C+E and C+E functions. Next to this partially overlapping expression pattern, class A and C genes are functionally antagonists, and C + E functions are also involved in floral determinacy (Coen and Meyerowitz, 1991; Ferrario et al., 2003; Goto et al., 2001; Pelaz et al., 2001). Additionally, the D functional genes act in ovule formation (Angenent et al., 1995).

In monocot species, despite many cases of significant differences in floral structure, the ABCDE model appears to be applicable, but with reservations (Nagasawa et al., 2003). Monocot genes showing homologous ABCDE functions have already been identified (reviewed by Fornara et al., 2003). Rice and maize are model species for flower development in grasses and the expression pattern of ABCDE genes is helping to clarify the organ homology between flowers from grasses and other species, considering that this topic has been a matter of discussion for a long time.

In bulbous species, such as hyacinth (*Hyacinthus orientalis*), lily (*Lilium* spp.), narcissus (*Narcissus pseudonarcissus*), and orchids (*Oncidium* sp. and *Dendrobium* sp.), MADS-box genes involved in flower development are also being isolated and functionally characterized (Li et al., 2002; Tzeng and Yang, 2001; Tzeng et al., 2002; Hsu and Yang, 2002; Yu and Goh, 2000). Liliaceae species show an interesting floral aspect with the perianth organs (sepals and petals) evolving into petal-like structures, receiving the designation of "tepals". This feature led to the proposition of a modified ABCDE model for the Liliaceae family members, with its B function being extended to the first whorl, in order to cope with petal characteristics of this organs (van Tunen et al., 1993).

Perspectives for reverse genetics of floral transcription factors based on VIGS

Overexpression of genes transcribing hpRNA of several genes involved in flower developmental pathways leads to specific and heritable silencing of the endogenous genes, resulting in null phenotypes in *Arabidopsis* (Chuang and Meyerowitz, 2000).

VIGS of a regulatory flower developmental gene was first accomplished in *N. benthamiana* with its *LEAFY*/*FLORICAULA* orthologue, *NFL* (Ratcliff et al., 2001), opening avenues for using this system for efficient characterization of floral gene functions.

Homeotic transcription factors, including the MADS-box genes involved in the ABCDE model, are perfectly suitable for assessing their function via VIGS, since they can instigate clear null phenotypes. They present a sequence structure conserved throughout evolution, that may permit studies of reverse genetics in heterologous systems, especially in model species in which VIGS is easily manipulated, such as *N. benthamiana* and, perhaps, rice.

Our hypothesis is that a VIGS vector carrying a fragment of a homeotic transcription factor would trigger transient silencing of its endogenous homologue and

induce phenotypes with homeotic transformations, if there is enough sequence identity between the genes involved.

Importantly, the sequence structure within members from a gene family can interfere with the proper specificity of silencing, since gene boxes with highly conserved sequences can provoke functional suppression not only of the target gene but also of other members of this family (Ratcliff et al., 2001), as confirmed by Immink et al. (1999) with a crossed cosuppression event between two MADS-box genes in petunia. For optimal results leading to a specific, effective and reliable gene silencing in a heterologous system, the establishment of the level of sequence homology required is pivotal information. It is generally assumed that 85% nucleotide identity would be the lowest limit for triggering the silencing mechanism, however, experimental evidence is being awaited.

Although some studies are still necessary to enable the use of VIGS in the functional identification of MADS-box genes in homo- or heterologous systems, the future seems promising and possibly in the near future this field will be open for speedy and easier functional characterization of the many genes from new species that await their function to be discovered or confirmed.

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EPILOGUE

The ABCDE model for lily

The early ABC model for floral organ development was proposed in the beginning of the 1990's, based on observations of floral homeotic mutants of *Arabidopsis thaliana* and *Antirrhinum majus* (Coen and Meyerowitz, 1991).

As soon as the early ABC model was proposed, van Tunen et al. (1993) postulated that, as flowers of members of the Liliaceae family show tepals instead of sepals and petals, they could present a distinct gene expression pattern of some genes of the model. This hypothesis was mainly alluding to the B function, of which the expression would have expanded towards the first whorl in order to provide petal appearance to the first whorl organs as well.

Lily species (*Lilium* spp.), despite their importance as ornamental flowers, had the first report about a molecular study on floral genes no earlier than the year 2000, when Theissen et al. (2000) sequenced several MADS-box genes from *L. regale* and located them phylogenetically among many other MADS-box genes derived from several species. As most of homeotic transcription factors involved in the ABCDE model encompass a MADS domain, this was the beginning of the flower development history in lily at its molecular level.

Following this first event, the molecular genetics of lily has progressed at a rapid pace. Several functional orthologues from *L. longiflorum* have been identified in the last two years, including a B type (*LMADS1*; Tzeng and Yang, 2001) and a putative D type gene (*LMADS2*; Tzeng et al., 2002).

This thesis contributes to this research area with the identification and characterisation of *L. longiflorum* genes that may hold the C and the E functions (*LLAG1* in chapter two and *LLSEP3* in chapter three, respectively) during floral organ development. Both genes were functionally characterised in the heterologous model species *Arabidopsis*.

Figure 1 shows the current state of the ABCDE model for flower development in *L. longiflorum* in comparison with the most studied reference, *Arabidopsis*. It can be noticed that the A function is still elusive in the species.

Our contribution to the genetic characterization of the ABCDE functions in *Lilium* is significant in fundamental and applied fields. Evolutionary and developmental biologists can use this information to assess some aspects of floral biology in monocots, and in the Liliaceae family particularly, such as protein

interaction patterns (within the species itself and even beyond the monocot class) and differences in gene promoter activity of transcription factors. On the other hand, molecular breeders can take advantage of the knowledge acquired on lily flower genetics and attempt to manipulate its floral morphology and flowering time, in order to generate novel and commercially interesting phenotypes.

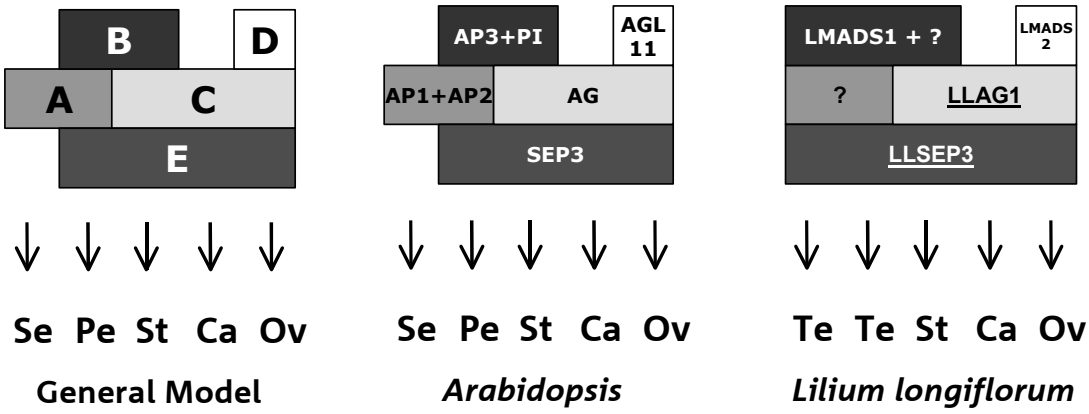

Figure 1. The ABCDE model for flower development**.** The current model states that five genetic functions act in an overlapping fashion in order to trigger the development of floral organs. Genes of each function were found in the model species *Arabidopsis*. In *Lilium longiflorum*, some of the ABCDE functions were already characterized. This thesis presents the characterization of *LLAG1* and *LLSEP3* genes, showing C and E functions, respectively.

Heterologous system for functional characterisation of lily genes

Given the methodological difficulties encountered in lily to genetic studies of flower morphology in a homologous system, such as its recalcitrance to genetic transformation and a long vegetative phase, we approached the functional characterisation of lily floral genes in a heterologous system, using the model species *Arabidopsis thaliana*.

Despite the easy manipulation of this model species, we met some obstacles during the characterization of *LLAG1*, which are described in the chapter four of this thesis.

Notwithstanding, the use of *Arabidopsis* as a heterologous system in flower development is not discouraged when the homologous system is not available or presents inconveniences. Given the evolutionary conservation of the ABCDE model and the extensive studies in flower development carried on in *Arabidopsis*, this species

is an appropriate system allowing (or speeding up) heterologous functional characterisation of floral genes.

Transformation system for lily

In our efforts to genetically transform lily, described in chapter five, we were able to generate three transgenic lily clones that were resistant to bialaphos in *in vitro* conditions via particle bombardment.

This transformation method has proven to be of low efficiency in lily and the current trend is towards *Agrobacterium* systems (Mercuri et al., 2003). However, our preliminary results indicated that this system is also recalcitrant and considerable input is still needed to increase its applicability in lily species.

The low number of transgenic clones recovered in the work reported in this chapter did not allow us to reach further goals, such as characterising the activity of a transcription factor and of a gene promoter from dicot species in lily. Nevertheless, given the recalcitrance of lily to transformation, obtaining three transgenic clones out of a single experiment is already a remarkable result.

We conclude, based on this work and on our further experience, that lily transformation is feasible, although the particle bombardment protocols currently available do not always provide us with reproducible transformation rates. Nevertheless, we suggest more investigation into methods based on *Agrobacterium*, since a consistent transformation method is already available for other Liliaceae species, such as onion and shallots (Zheng et al., 2001).

Novel floral mutants of lily and new challenges

Two types of homeotic floral mutants were found in lily during our studies. Some double flowers, i.e. *agamous*-like homeotic lily mutants, are commercially available and the correlated phenotype is found in several species. It is clear that it involves the C function of the ABCDE model (Roeder and Yanofsky, 2001), due to its coupled abnormalities (homeotic mutation and loss of floral determinacy). It would be very interesting to investigate at what level this function is misregulated. It is possible that the *LLAG1* gene is involved in the process, since it showed a C function when expressed in *Arabidopsis* (chapter two).

We also speculated about a novel homeotic floral phenotype found in lily and never observed in other species, which challenges the current ABCDE model because it presents a homeotic change in only one whorl. In this conceptual chapter, we raised

several hypotheses that may fascinate researchers and encourage them to find the answers and open new doors for manipulation of floral morphology.

Virus-induced gene silencing system

The advent of the virus-induced gene silencing (VIGS) system brought insights in new ways of studying functions of homeotic genes involved in flower development.

In chapter seven of this thesis we described the genetics, molecular mechanisms, and methodological details of the VIGS system. We also speculate on the possibility of assessing the functions of monocot genes, and particularly those from lily, in a heterologous model system, such as *Nicotiana benthamiana* and rice.

Preliminary results indicated that a gene fragment of *phytoene desaturase* derived from lily is able to elicit some degree of silencing in *N. benthamiana*. We hypothesised that floral homeotic functions are also capable to be silenced by the same mechanism.

The information contained in this section will help to establish an approach to test our hypothesis that a model heterologous system, such as *N. benthamiana* or rice, can be used to ascribe gene function of floral homeotic transcription factors from monocot species.

Prospects for research on flower development of lily

Analyses of gene functions involved in lily flower development and generation of useful information on the molecular breeding potential of this species were the main objectives of the work described in this thesis.

We could identify putative players in flower development of lily, we found interesting and novel homeotic floral mutants, and generated transgenic plants. Moreover, we also evaluated the possibility of using VIGS as a promising method for functionally characterise homeotic genes from monocots in a heterologous system as well as the homologous species, and raised hypotheses on the homeotic floral mutants that we found.

Interesting topics for further investigation on flower development of lily could be on protein-protein interactions of transcription factors (intra-specifically and also between lily and dicot proteins); a further characterisation of the transcriptional profile of flower-specific genes could be assessed by *in situ* hybridisation; the functional characterisation of lily genes could be tested with VIGS in a heterologous model

system, such as *N. benthamiana* or rice; and the *bona fide* domain of B functional genes could be better characterised in lily, in order to further substantiate the proposed dislocated model.

The field for studying the molecular aspects of lily flower development is wide open and the future may uncover very interesting aspects that will produce new tools for ornamental breeders as well as reveal particular features of monocots and the Liliaceae plant family.

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SUMMARY

Lily *(Lilium* spp.) is among the most traditional and beloved ornamental flowers worldwide. The genus *Lilium* comprises almost one hundred species, among which is the primary subject of our research, described in this thesis, the species *Lilium longiflorum* (Thunb.), known as trumpet lily or Easter lily.

Despite the great economic importance of ornamental lily species, little is known about its biology at the molecular level so far. In a time when two genomes are fully sequenced, *Arabidopsis thaliana* and *Oryza sativa*, only a few genes have been characterized in *Lilium* spp. yet. Possible reasons for this are discussed throughout this thesis.

This work intends to be a contribution to bridging the fundamental research concerning transcription factors involved in development of flower morphology in model species and the applied objectives of molecular breeding for manipulating flower morphology, endeavouring to create new cultivars with specific and novel features, more specifically in *Lilium* spp.

The ABC model for floral development was proposed more than 10 years ago and since then many studies have been performed in model species, such as *Arabidopsis thaliana*, *Antirrhinum majus*, petunia and many other species in order to confirm this model. This investigation has led to additional information on flower development and to more complex molecular models.

In the first chapter of this thesis, notions of molecular floral development, the difficulties of working with molecular biology of lily, the state-of-the-art in lily transformation are introduced, as well as general overviews of transcription factors, MADS-box genes, the ABCDE model for flower development and functional characterization of genes in heterologous systems. These concepts will guide the reader throughout the work we present here.

AGAMOUS (*AG*) is the only C type gene found in *Arabidopsis* and it is responsible for stamen and carpel development as well as floral determinacy. In the second chapter, we describe the isolation of *LLAG1*, a putative *AG* orthologue from lily (*L. longiflorum*) by screening a cDNA library derived from developing floral buds. The deduced amino acid sequence of *LLAG1* revealed the MIKC structure and a high homology in the MADS-box among *AG* and other orthologues. Phylogenetic analysis indicated close relationship between *LLAG1* and *AG* orthologues from monocot species. Spatial expression data showed *LLAG1* transcripts exclusively in stamens and

carpels, constituting the C domain of the ABC model. Functional analysis was carried out in *Arabidopsis* by overexpression of *LLAG1* driven by the CaMV 35S promoter. Transformed plants showed homeotic changes in the two outer floral whorls with some plants having the second whorl completely converted into stamens. Altogether, these data indicate a functional relationship between *LLAG1* and *AG*.

SEPALLATA3 (*SEP3*) is a MADS-box homeotic gene possibly determining the E function in the ABCDE model. This function is essential for proper development of petals, stamens and carpels. In order to gain further information on lily (*Lilium longiflorum*) flower development at the molecular level, the cDNA library constructed from developing floral buds was screened again and our findings are reported in the chapter three. A clone (*LLSEP3*) was isolated with high similarity to the *SEP3* transcription factor from *Arabidopsis*. *LLSEP3* belongs to the *AGL2* subfamily of MADS-box genes and shares its closest relationships with *DOMADS1* and *OM1*, from the orchid species *Dendrobium grex* and *Aranda deborah*, respectively. Expression analysis by Northern hybridisation showed that *LLSEP3* was expressed throughout lily flower development and in tepals, stamens and carpel tissues of mature flowers, whereas no expression was detected in leaves. Overexpression of *LLSEP3* in *Arabidopsis* under the CaMV35S promoter induced early flowering but did not induce any floral homeotic changes, which is in accordance with the effect of *SEP3* overexpression in this species. Altogether, these data are consistent with the putative role of *LLSEP3* as an E functional gene in lily flower development.

Drawbacks found during our work on functional characterisation of *LLAG1*, by means of complementing the *agamous* mutant of *Arabidopsis thaliana* are described and critically discussed in chapter four. Such difficulties are, on the one hand, the nature of the *AGAMOUS* gene, of which the loss of function induces sterility and, on the other hand, the unavailability of the defective *ag-1* allele in another *Arabidopsis* background than the *Landsberg erecta* ecotype, which is recognisably difficult to transform by the floral dip method. Even though we did not manage to complement the *AG* function with *LLAG1* in a defective *ag* genotype so far, we could observe clear floral homeotic changes in those *Arabidopsis* plants ectopically overexpressing *LLAG1*, which together with our data on sequence identities and expression profile described in the previous chapter of this thesis, indicated that *LLAG1* is a strong candidate to control the C function in *L. longiflorum*.

This work also contributes towards the improvement of lily transformation procedures. In the chapter five we describe a transformation of bulblet slices by particle bombardment using a vector carrying the *Arabidopsis SUPERMAN* gene driven by the petunia flower-specific *FLORAL BINDING PROTEIN 1* promoter and

the bialaphos resistance gene *phosphinothricin acetyltransferase* under the *CaMV35S* promoter. Our intentions were improving the transformation parameters for lily transformation in order to reach higher efficiency, and creating novel phenotypes in lily flowers using transcription factors originating from dicot plants. We were capable of obtaining transgenic lines expressing *in vitro* resistance to bialaphos. The transgenic plants were transferred to the greenhouse, grown and monitored for two flowering seasons. Flowers derived from these plants appeared normal and indistinguishable from wild-type flowers and the possible reasons for this are currently under investigation.

Homeotic changes in floral organs of lily (*Lilium* spp.) are described in chapter six. Usually, lily flowers show similar organs in their first and second whorls called tepals. They constitute the appealing and colourful features determining flower appearance. Stamens and the pistil appear as the third and fourth whorls, respectively. A double lily flower shows replacement of stamens by tepals and of its carpel by a new flower in a reiterated manner, similar to what is seen in the *agamous* mutant of *Arabidopsis*. A novel floral phenotype of lily, denominated *festiva* here, has never been reported in other species so far and shows a complete homeotic change of stamens into tepals, but keeps the carpel identity. We tried to explain these phenotypes taking into consideration all the evidence on the genetic mechanisms involved in flower development gathered over the last 15 years. This work launches challenges and encouragement for exploiting the molecular mechanisms involved in flower development of lily.

Virus-induced gene silencing (VIGS) system has shown to be of great potential in reverse plant genetics. Advantages of VIGS over other approaches, such as T-DNA or transposon tagging, include the circumvention of plant transformation, methodological simplicity and robustness, and speedy results. These features enable VIGS as an alternative instrument in functional genomics, even in a high throughput fashion. The system is already well established in *Nicotiana benthamiana*, but efforts are being addressed to improve VIGS in other species, including monocots. Current research is focussed on unravelling the silencing mechanisms of post-transcriptional gene silencing (PTGS) and VIGS, as well as finding novel viral vectors in order to broaden the host species spectrum. In chapter seven, we discuss the advantages of using VIGS to assess gene functions in plants. We address the molecular mechanisms involved in the process, present the available methodological elements such as vectors, inoculation procedures, and we show examples in which the system was applied successfully to characterize gene function in plants. Moreover, we analyse the potential

application of VIGS in assessing genetic function of floral transcription factors from monocots.

Analyses of gene functions involved in lily flower development and generation of useful information on the molecular breeding potential of this species were the main objectives of the work described in this thesis.

The field for studying the molecular aspects of lily flower development is now wide open and the future may uncover very interesting aspects that will produce new tools for ornamental breeders as well as reveal particular features of monocots and the Liliaceae plant family.

SAMENVATTING

De lelie (*Lilium spp.)* behoort tot de meest traditionele en geliefde bloemen ter wereld. Het geslacht *Lilium* omvat bijna honderd soorten, waaronder het voornaamste onderwerp van het onderzoek in dit proefschrift, *Lilium longiflorum* (Thunb.), ook wel bekend als 'trumpet' of 'Easter' lelie.

Ondanks het grote economische belang van de sierlijke lelie, is nog slechts weinig moleculaire kennis beschikbaar. In het tijdperk, waarin van twee plantengenomen de volledige basen(nucleotiden)volgorde is bepaald, namelijk van *Arabidopsis thaliana* oftewel de zandraket en van *Oryza sativa*, rijst, zijn er in lelie (*Lilium* spp.) slechts enkele genen gekarakteriseerd. Mogelijke redenen hiervoor worden op diverse plaatsen in dit proefschrift bediscussieerd.

Dit proefschrift stelt zich tot doel om bij te dragen aan het slaan van een brug tussen het fundamentele onderzoek gericht op transcriptiefactoren betrokken bij de bloemontwikkeling in modelsoorten en de meer toegepaste doelstellingen van moleculaire veredeling gericht op het beïnvloeden van de bloemvorm teneinde nieuwe rassen te kunnen creëren met specifieke en nieuwe kenmerken, met name in lelie.

Het ABC model voor bloemontwikkeling werd reeds meer dan tien jaar geleden voor het eerst opgesteld en sindsdien is er veel onderzoek uitgevoerd in modelgewassen, zoals *Arabidopsis thaliana, Antirrhinum majus,* petunia en diverse andere soorten om dit model te bevestigen. Dit werk heeft veel aanvullende informatie opgeleverd op het gebied van bloemontwikkeling en heeft geleid tot veel complexere moleculaire modellen (het ABCDE model).

In het eerste hoofdstuk van dit proefschrift, denkbeelden over moleculaire aspecten van bloemontwikkeling, worden verschillende facetten geïntroduceerd, die het werken aan de moleculaire biologie van lelie bepaald niet hebben vergemakkelijkt. Daarnaast geeft dit hoofdstuk een overzicht van de stand van zaken met betrekking tot de mogelijkheden van genetische modificatie van lelie en van functionele karakterisering van genen in heterologe systemen plus een algemeen overzicht van transcriptiefactoren, MADS-box genen en het ABCDE model voor bloemvorming. Deze introductie zal de lezer behulpzaam zijn bij het begrijpen van het onderzoek dat hierna wordt gepresenteerd.

AGAMOUS (AG) is het enige C type gen dat gevonden is in *Arabidopsis;* het is verantwoordelijk voor de ontwikkeling van meeldraden en vruchtbeginsel(s) en voor de bepaling van de aard van het orgaan als zijnde 'bloem'. In het tweede hoofdstuk

wordt de isolatie van *LLAG1* beschreven, de hypothetische *AG* ortholoog (= een gen met een vergelijkbare functie) van lelie (*Lilium longiflorum)*. Dit is gebeurd door een cDNA bank door te lichten die was gemaakt van jonge, ontwikkelende bloemknoppen. Uit de basenvolgorde van *LLAG1* is vervolgens de aminozuurvolgorde afgeleid. Deze aminozuurvolgorde gaf qua eiwitorganisatie de verwachte MIKC domeinen te zien en ook een hoge mate van overeenkomstigheid (homologie) in de z.g. MADS box, één van die eerder genoemde domeinen. Een fylogenetische analyse gaf aan dat er een nauwe verwantschap bestaat tussen *LLAG1* en *AG* orthologen van andere monocotyle soorten. Onderzoek naar de ruimtelijke expressie toonde aan dat de *LLAG1* transcripten uitsluitend voorkwamen in de meeldraden en vruchtbeginsels, zijnde het C domein van het ABC model. De functionele analyse is uitgevoerd in *Arabidopsis* door het *LLAG1* gen tot overexpressie te brengen met behulp van de CaMV 35S promotor. Transgene planten vertoonden homeotische veranderingen in de twee buitenste ringen van bloemorganen, waarbij in sommige plantjes de tweede ring, normaal bestaand uit kroonbladeren, volledig was omgevormd tot meeldraden. Alles bij elkaar genomen suggereren deze resultaten een functionele verwantschap tussen *LLAG1* en *AG*.

SEPALLATA3 (SEP3) is een MADS-box homeotisch gen dat mogelijk betrokken is bij de E functie in het ABCDE model. Deze functie is essentieel voor de juiste ontwikkeling van de kroonbladeren, meeldraden en het vruchtbeginsel. Teneinde meer inzicht te krijgen in de moleculaire achtergronden van bloemontwikkeling in lelie *(Lilium longiflorum)* is de cDNA bank die gemaakt was van jonge bloemknoppen, opnieuw gescreend, o.a. voor *SEP*-homologen; de resultaten hiervan worden vermeld in hoofdstuk drie. Een kloon (*LLSEP3)* die in hoge mate overeenkwam met de *SEP3* transcriptiefactor van *Arabidopsis* werd geïsoleerd. *LLSEP3* behoort tot de *AGL2* sub-familie van MADS-box genen en vertoont de meest nauwe verwantschap met *DOMADS1* en *OM1*, afkomstig van de orchideeën *Dendrobium grex* en *Aranda deborah*, respectievelijk. Expressie analyse door middel van Northern hybridisatie gaf aan dat *LLSEP3* tot expressie komt gedurende alle ontwikkelingsstadia van de leliebloem en in de bloembladeren, meeldraden en het vruchtbeginsel van de uiteindelijke bloem, terwijl geen expressie gedetecteerd kon worden in de bladeren. Overexpressie van *LLSEP3* in *Arabidopsis* onder controle van de CaMV 35S promotor induceerde vervroegde bloei, maar geen homeotische veranderingen. Dit is in overeenstemming met het verwachte effect van *SEP3* overexpressie in deze soort. Alles bij elkaar zijn deze resultaten consistent met een mogelijke rol van *LLSEP3* als gen met een E functie in bloemvorming bij lelie.

Hoofdstuk vier beschrijft enkele moeilijkheden, die bij de functionele karakterisering van *LLAG1* door middel van complementatie van de *agamous* mutant

van *Arabidopsis thaliana* aan het licht kwamen. Deze problemen zijn verbonden met de aard van het *AGAMOUS* gen, waarvan functie-uitval steriliteit induceert; daarnaast bleek het defectieve *ag-1* allel alleen maar beschikbaar in het *Landsberg erecta* ecotype van *Arabidopsis*. Dit ecotype is erg moeilijk te transformeren via de z.g. 'floral dip' methode. Hoewel het dus niet mogelijk is gebleken verder bewijs voor de C functie van *LLAG1* te verschaffen door complementatie van *ag*-defectieve mutanten in *Arabidopsis* zijn de resultaten gepresenteerd in hoofdstuk twee van dit proefschrift een sterke indicatie voor de rol van *LLAG1* als bepaler van de C functie in *L. longiflorum.*

Het onderhavige onderzoek heeft ook bijgedragen aan de verbetering van transformatiemethoden in lelie. In hoofdstuk vijf wordt de transformatie van bolschubjes beschreven door middel van 'particle bombardment', waarbij een vector is gebruikt die het *Arabidopsis SUPERMAN* gen bevatte gereguleerd door de bloem-specifieke *FLORAL BINDING PROTEIN 1* promotor van petunia en daarnaast het herbicide-resistentie gen, *phosphinothricin acetyltransferase*, gereguleerd door de CaMV 35S promotor. Het doel was om parameters van belang in de transformatie van lelie waarmee een hogere efficiëntie bereikt kon worden en om nieuwe fenotypen te creëren in lelie bloemen door gebruik te maken van transcriptiefactoren afkomstig van dicotyle planten. Diverse transgene planten zijn verkregen, die *in vitro* resistent waren tegen het herbicide bialaphos; enkele hiervan zijn d.m.v. PCR positief gebleken voor het *SUPERMAN* gen. De transgene planten zijn naar de kas overgebracht en opgekweekt. Gedurende twee bloeiseizoenen zijn de planten gevolgd, maar in tegenstelling tot de verwachting zagen de bloemen van deze planten er normaal uit en waren zij niet te onderscheiden van wild-type bloemen. Mogelijke verklaringen hiervoor worden op dit moment onderzocht.

Natuurlijke homeotische veranderingen in de bloemorganen van lelie (*Lilium* spp.) worden beschreven in hoofdstuk zes. Normaliter zijn de organen in de eerste en tweede ring bij leliebloemen, nl. de kelk en de kroon, qua uiterlijk aan elkaar gelijk. Zij worden tepalen genoemd en vormen de aantrekkelijke en kleurrijke kenmerken van de bloem. De meeldraden en de stijl vormen de derde en vierde ring respectievelijk. Bij bestaande, dubbelbloemige lelievariëteiten zijn de meeldraden vervangen door tepalen en het vruchtbeginsel door een nieuwe bloem (in een bloem dus). Dit fenotype komt overeen met het fenotype dat in *Arabidopsis* bij de *agamous* mutant is beschreven en valt te verklaren met het ABCDE model. Een nieuw bloemtype in lelie, in dit proefschrift voor het eerst benoemd als *festiva,* is nog nooit beschreven in een andere soort. Dit type vertoont de volledige homeotische omzetting van de meeldraden in tepalen, maar met behoud van de identiteit van het vruchtbeginsel. In dit hoofdstuk

wordt een poging gedaan dit bloemtype te verklaren uitgaande van al de kennis die in de loop van de laatste vijftien jaar verzameld is met betrekking tot de genetische mechanismen die ten grondslag liggen aan bloemontwikkeling.

In hoofdstuk zeven worden de voor- en nadelen bediscussieerd van het gebruik van 'Virus-induced gene silencing' (VIGS) bij het bepalen van genfuncties in planten. Het hoofdstuk behandelt mogelijke moleculaire mechanismen, geeft een overzicht van de beschikbare elementen zoals vectoren en inoculatie procedures en geeft voorbeelden waarin het systeem succesvol is toegepast in de identificatie van genfuncties in planten. Tot slot wordt de mogelijke toepassing van VIGS bij het bepalen van de functie van transcriptiefactoren in bloemen van monocotylen geëvalueerd. VIGS biedt grote mogelijkheden als instrument binnen functionele genanalyse. Voordelen van VIGS boven andere 'knock-out' of uitvalbenaderingen, zoals T-DNA of transposon inserties, zijn gelegen in het vermijden van transformatie, in methodologische eenvoud, robuustheid en snelheid. Deze kenmerken maken van VIGS een waardevol alternatief, ook in een opgeschaalde opzet. Het systeem is reeds goed toepasbaar bij *Nicotiana benthamiana*, maar veel inspanningen zijn er recent op gericht om VIGS breder inzetbaar te maken in veel meer soorten, waaronder monocotylen. Internationaal is het onderzoek er op gericht om het werkingsmechanisme van het z.g. 'gene silencing' te ontrafelen, maar ook om nieuwe virale vectoren te ontwerpen die kunnen bijdragen aan de uitbreiding van het gastheerspectrum.

Dit onderzoek heeft uitdagingen en aanmoedigingen opgeleverd om de moleculaire mechanismen betrokken bij bloemvorming in lelie verder te verkennen en te exploiteren. Hiermee is aan de oorspronkelijke doelstellingen van het in 1999 gestarte onderzoek voldaan. Het is nu duidelijk dat er grote mogelijkheden zijn om de moleculaire aspecten van bloemontwikkeling in lelie te bestuderen. In de toekomst zullen er ongetwijfeld nog meer methoden beschikbaar komen die de lelieveredelaar kan gebruiken en zullen er meer specifieke kenmerken van monocotylen en meer speciaal van de *Liliaceae* familie aan het licht komen.

RESUMO

O lírio *(Lilium* spp.) está entre as espécies ornamentais mais tradicionais e cultivadas do mundo. O gênero *Lilium* engloba quase cem espécies, entre as quais está o objetivo principal dos estudos descritos nesta tese, a espécie *L. longiflorum* (Thunb.), conhecida popularmente como lírio branco.

Apesar da grande importância econômica das espécies ornamentais de lírio, pouco se tem pesquisado a sua biologia molecular. Numa época em que dois genomas vegetais estão completamente seqüenciados, *Arabidopsis thaliana* e *Oryza sativa*, poucos genes foram caracterizados em *Lilium* spp. As possíveis razões para isso estão discutidas nesta tese.

Este presente trabalho pretende ser um elo de ligação entre a pesquisa fundamental dos fatores de transcrição envolvidos no desenvolvimento da morfologia floral em espécies-modelos e os objetivos aplicados do melhoramento molecular para a manipulação da morfologia floral do lírio, no intuito de criar novas variedades com características inéditas e específicas.

O modelo ABC para desenvolvimento floral foi proposto há mais de 10 anos e desde então muitos estudos foram realizados em espécies-modelos, como *Arabidopsis thaliana*, *Antirrhinum majus* (boca-de-leão), petúnia e muitas outras espécies, para confirmar o modelo proposto. Esta intensa atividade científica em torno do desenvolvimento molecular da flor levou ao surgimento de modelos moleculares mais complexos, como o modelo ABCDE de desenvolvimento floral e o modelo quaternário de complexação de fatores de transcrição.

No primeiro capítulo desta tese são apresentadas noções do desenvolvimento floral no nível molecular, as dificuldades encontradas no trabalho com a biologia molecular do lírio (*L. longiflorum*) e a situação técnica atual da transformação da espécie, bem como uma visão geral dos fatores de transcrição, dos genes da família MADS-box, do modelo ABCDE para desenvolvimento floral e da caracterização gênica funcional em sistemas heterólogos de plantas-modelos.

AGAMOUS (*AG*) é o único gene do tipo C encontrado em *Arabidopsis*, sendo responsável pelo desenvolvimento dos estames e do carpelo, bem como pelo hábito determinado do desenvolvimento floral. O segundo capítulo desta tese descreve o isolamento do *LLAG1*, um ortólogo putativo do gene *AG* em lírio (*L. longiflorum*) pelo escrutínio de uma biblioteca de cDNA obtida a partir de botões florais em desenvolvimento. A seqüência deduzida de aminoácidos do *LLAG1* revelou um gene

MADS-box com a estrutura MIKC e alta homologia com o gene *AG* e outros ortólogos. Análises filogenéticas indicaram relação mais próxima entre *LLAG1* e os ortólogos do *AG* de espécies monocotiledôneas. Análise da expressão gênica de *LLAG1* revelou transcrição ativa exclusivamente nos estames e carpelos, o que constitui o domínio C do modelo ABC. A análise gênica funcional foi conduzida em *Arabidopsis* pela superexpressão de *LLAG1* dirigida pelo promotor CaMV35S. As plantas transformadas mostraram mudanças homeóticas nos dois verticilos florais do perianto, com algumas plantas tendo o segundo verticilo completamente convertido em estames. Os dados apresentados e discutidos nesse capítulo indicam a relação funcional entre *LLAG1* e *AG*.

SEPALLATA3 (*SEP3*) é um gene homeótico MADS-box que possivelmente determina a função E no modelo ABCDE. Esta função é essencial para o desenvolvimento normal de pétalas, estames e carpelos. No intuito de obter mais informações sobre o desenvolvimento molecular da flor do lírio (*L. longiflorum*), a biblioteca de cDNA de botões florais em desenvolvimento foi escrutinada mais uma vez e os resultados estão relatados no capítulo três da presente tese. Um clone (*LLSEP3*) com alta similaridade ao fator de transcrição *SEP3* de *Arabidopsis* foi isolado. *LLSEP3* pertence à subfamília *AGL2* dos genes MADS-box e apresenta estreita relação com *DOMADS1* e *OM1*, genes das orquídeas *Dendrobium grex* e *Aranda deborah*, respectivamente. A análise da expressão gênica por hibridação northern mostrou que *LLSEP3* é expresso durante todo o desenvolvimento floral, estando presente também em tecidos de tépalas, estames e carpelos de flores maduras, enquanto sua expressão não foi detectada em folhas. A superexpressão de *LLSEP3* dirigida pelo promotor CaMV35S em *Arabidopsis* induziu florescimento precoce, mas não coordenou mudanças homeóticas nos órgãos florais, o que está de acordo com o efeito da superexpressão de *SEP3* nesta mesma espécie. A análise conjunta dos dados é consistente com o possível papel de *LLSEP3* como um gene funcional do tipo E no desenvolvimento floral em lírio.

As dificuldades encontradas durante o trabalho de caracterização funcional do *LLAG1*, por meios de complementação do mutante *agamous* de *Arabidopsis thaliana* estão descritos criticamente no capítulo quatro desta tese. Tais dificuldades são devido, por um lado, à natureza do gene *AGAMOUS*, cuja perda funcional induz esterilidade e, por outro lado, à indisponibilidade do alelo defectivo *ag-1* em um ecótipo de *Arabidopsis* diferente da *Landsberg erecta*, que apresenta baixos índices de transformação pelo método de mergulho floral. Apesar de não ter-se alcançado o complemento da função *AG* com *LLAG1* no genótipo defectivo homozigoto *ag* até o momento, pôde-se observar claras mudanças homeóticas florais nas plantas de tipo

selvagem de *Arabidopsis* superexpressando ectopicamente *LLAG1*, o que junto com os dados do capítulo dois desta tese sobre as identidades de seqüência e o perfil de expressão gênica, indicam que *LLAG1* é um forte candidato a ter controle sobre a função C em *L. longiflorum*.

Este trabalho também se propõe a contribuir com o melhoramento dos procedimentos metodológicos de transformação do lírio. No capítulo cinco desta tese, relata-se a transformação de segmentos de bulbilhos por bombardeamento de partículas com um vetor carregando o gene *SUPERMAN* de *Arabidopsis* sob controle do promotor *FLORAL BINDING PROTEIN 1*, específico da flor de petúnia, e o gene de resistência ao bialafós *acetiltranferase da fosfinotricina*, sob controle do promotor *CaMV35S*. Objetivou, assim, estudar os parâmetros de transformação do lírio no intuito de se obter uma maior eficiência metodológica, além de se produzir novos fenótipos nas flores de lírio usando-se fatores de transcrição originários de espécies dicotiledôneas. Neste trabalho, foram obtidas linhagens transgênicas de lírio expressando resistência *in vitro* ao bialafós. As plantas transgênicas foram transferidas à casa de vegetação e monitoradas por dois períodos de florescimento, mas as flores derivadas destas plantas mostraram-se com fenótipo inalterado, indistingüíveis das flores de lírio não transformadas. As possíveis razões disto estão atualmente sob investigação.

Dois exemplos de mudanças homeóticas nas flores do lírio (*Lilium* spp.) estão descritos no capítulo seis. Normalmente as flores de lírio apresentam órgãos similares nos seus dois verticilos externos, denominados tépalas, apresentando um forte apelo estético. Os estames e o pistilo aparecem como terceiro e quarto verticilos, respectivamente. Uma flor dupla de lírio apresenta tépalas no lugar dos estames e uma nova flor no lugar do pistilo, numa maneira reiterada, similar ao fenótipo visto no mutante *agamous* de *Arabidopsis*. Um novo fenótipo floral encontrado em lírio, denominado *festiva*, nunca foi relatado anteriormente em espécies-modelos e apresenta mudança homeótica completa dos estames em tépalas, enquanto guarda a identidade do pistilo no verticilo mais interno. Explicações a estes fenótipos de lírio foram propostas, levando-se em consideração as evidências nos mecanismos genéticos envolvidos no desenvolvimento floral obtidos nos últimos quinze anos. Este trabalho lança desafios e encoraja a exploração dos mecanismos moleculares envolvidos no desenvolvimento floral de lírio.

O sistema de silenciamento gênico induzido por vírus (*VIGS* ou *virus-induced gene silencing*) apresenta enorme potencial na genética reversa de plantas. As vantagens do sistema VIGS sobre outros métodos, tais como T-DNA ou *transposon tagging*, incluem a não utilização de transformação, a simplicidade e robustez

metodológica, além da rapidez de obtenção dos resultados. Estas características aludem ao sistema VIGS como um instrumento alternativo na genômica funcional, mesmo quando experimentos são desejados em larga escala. Este sistema já está bem estabelecido em *Nicotiana benthamiana*, mas esforços estão sendo atualmente alocados na melhoria do sistema VIGS em outras espécies, incluindo monocotiledôneas. A pesquisa neste campo tem-se focado atualmente em revelar os mecanismos do VIGS e do silenciamento gênico pós-transcricional (PTGS ou *post-transcriptional gene silencing*), bem como encontrar novos vetores virais no intuito de aumentar o espectro de espécies hospedeiras. No capítulo sete, discutem-se as vantagens de se utilizar o sistema VIGS para acessar funções gênicas em plantas. Apresentam-se os mecanismos moleculares envolvidos no processo, os elementos metodológicos disponíveis como vetores, procedimentos de inoculação, com exemplos nos quais o sistema foi aplicado com sucesso na caracterização da função de genes de plantas. Ademais, analisa-se o potencial da aplicação do sistema VIGS na caracterização funcional de fatores de transcrição envolvidos no florescimento de espécies de monocotiledôneas.

As análises de funções gênicas envolvidas no desenvolvimento floral de lírio e a geração de informações úteis no melhoramento molecular potencial deste espécie foram os principais objetivos do trabalho descrito nesta tese.

O campo de estudos dos aspectos moleculares do desenvolvimento floral de lírio está agora de portas abertas e o futuro poderá trazer aspectos muito interessantes que contribuam para levar novas ferramentas aos melhoristas de espécies ornamentais, bem como revelar características particulares do florescimento das monocotiledôneas e da família Liliaceae.

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Vagner Augusto Benedito was born in Osasco, Brazil, on January 5th 1974. Soon he moved to Americana where he started the primary school in the Antonio Zanaga II district at "E.E.P.G. Prof. Clarice Costa Conti". In 1989, he started the high school in Dracena together with the Agricultural Technical studies. His undergraduate studies in Agronomy were followed in "Universidade Federal de Viçosa" (UFV), where he discovered Science especially through Botany, Genetics, Statistics, Biochemistry and Molecular Biology. There he participated in the Scientific Initiation Program from the CNPq (the Brazilian Council for Research and Development), going to many scientific and politic congresses throughout the country and, in 1995 he won a national prize from SBPC (the Brazilian Society for Progress of Science) for his work with molecular biology of soybean, under the supervision of Prof. Dr. Elizabeth Fontes. In 1997 he finished his undergraduate studies and initiated a M.Sc. program on Horticulture at "Escola Superior the Agricultura Luiz de Queiroz" (ESALQ), a campus of the "Universidade de São Paulo" (USP). His M.Sc. dissertation involved protoplast fusions and the regeneration of tetraploid citrus. After getting the degree in 1999, he moved to Wageningen, the Netherlands, and started a Ph.D. program on Plant Molecular Biology at Wageningen University (WU), developing his investigations at Plant Research International (PRI). He characterised important transcription factors involved in flower development of lily, under the sponsorship of Brazilian people through CNPq.

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