Contents

1 Introduction

1.1 SBP-box Genes represent a major family of plant specific transcription factors

Development is based on the cellular capacity for differential gene expression and is often controlled by transcription factors acting as switches of regulatory cascades (Riechmann et al., 2000).

Transcription factors are usually defined as proteins that show sequence specific DNA binding capacity and are able to activate or repress the transcription of target genes.

In *Arabidopsis thaliana* approximately 6% of its estimated total number of genes code for transcriptional regulators (Ratcliffe and Riechmann, 2002). For comparison, in *C. elegans* only 3.5%, in *D. melanogaster* 4.6% and in humans 4.6-6.6% of all genes encode for transcription factors (Ratcliffe et al., 2002).

Analysis of the *Arabidopsis* genome revealed 49 transcription factor families according to their DNA binding domain (Ratcliffe et al., 2002; Guo et al., 2005).

One of these is formed by the *SQUAMOSA PROMOTER BINDING PROTEIN*-box genes, a diverse family defined by harboring a highly conserved DNA binding domain of 76 amino acid residues, termed the SBP-domain. They are found exclusively in green plants, from the single-celled *Chlamydomonas* to multicellular higher plants like *Arabidopsis*. Until now *SBP*-box genes have not been identified in prokaryotes, fungi or animals.

SBP-domain proteins were initially isolated from *Antirrhinum majus* by their capacity to interact *in vitro* with a promoter sequence element of the floral meristem identity gene *SQUAMOSA,* the presumed *Antirrhinum* ortholog of the *Arabidopsis* gene *APETALA1* (*AP1*), (Klein et al., 1996).

1.2 The *SPL* gene family in *Arabidopsis thaliana*

In order to study the role of SBP-box genes in plant development, Cardon and co-workers initiated a search for homologous genes of the *A. majus SBPs*, *SBP1* and *SBP2* in *Arabidopsis thaliana*. Initially 12 homologues were identified, named *SPL* genes for *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE* and numbered sequentially (Cardon et al., 1999). After completion of the sequencing of the *Arabidopsis* genome five more *SPL* genes could be added such that today 17 members of the SBP-box gene family are known in *A. thaliana*.

The whole family in *Arabidopsis* can be divided into subfamilies, based on genomic organization and sequence similarities. *SPL1, SPL7, SPL12, SPL14* and *SPL16* form one subfamily representing the largest and most complex members of the gene family. These

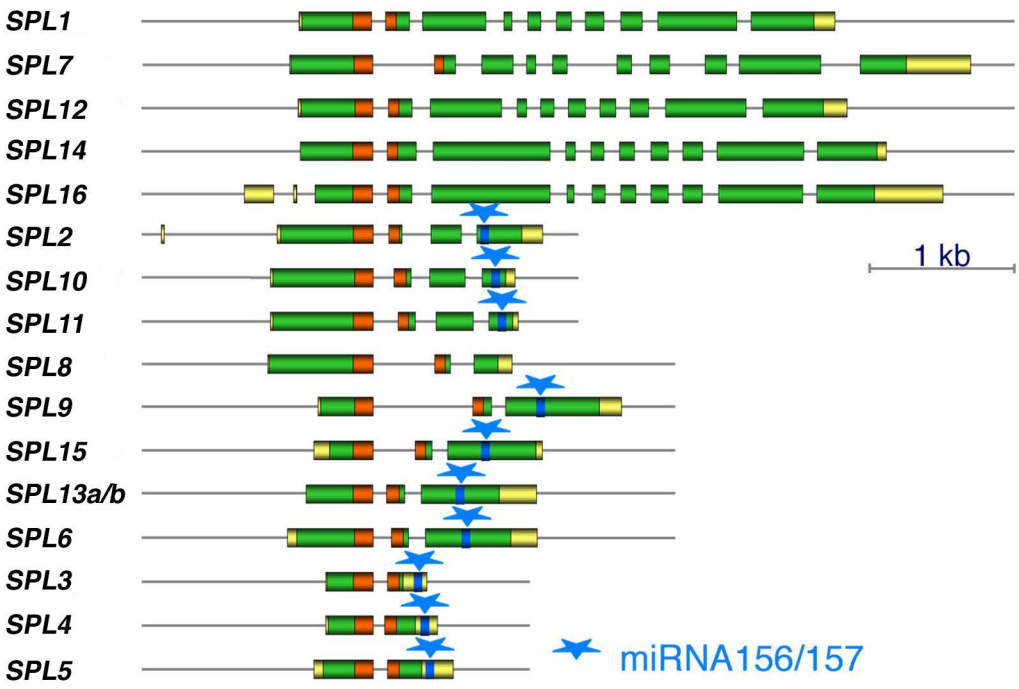

Figure 1.1.: The *SPL* gene family in *Arabidopsis thaliana*.
The *SPL* gene family in Arabidopsis consists of 17 members and can be divided into three subfamilies. The largest genes *SPL1*, *SPL7*, *SPL12*, *SPL14* and *SPL16* form one subfamily. The middle sized genes *SPL2*, *SPL10*, *SPL11*, *SPL8* , *SPL9*, *SPL15* as well as *SPL13a* and *SPL13b* form a second subfamily. The smallest genes, *SPL3*, *SPL4* and *SPL5*, can be grouped in a third subfamily. With the exception of *SPL8*, all middle and small sized genes are targets of the miRNA156 and the miRNA157. *SPL3*, *SPL4* and *SPL5* have the MiRNA recognition site in their 3' UTR whereas the other MiRNA targets have their recognition site located within the coding part of the last exon.
Green boxes indicate exons; red boxes indicate the SBP-box (split by an intron); yellow boxes indicate UTR's; blue boxes and blue stars indicate the miRNA156/157 recognition site.

genes are also characterized by the fact that they are expressed largely constitutively throughout plant development (Cardon et al., 1999; Schmid et al., 2005).

In contrast, the mid-sized genes (i.e. *SPL2, SPL6, SPL8, SPL9, SPL10, SPL11, SPL13a, SPL13b* and *SPL15*) as well as the small genes (i.e. *SPL3, SPL4* and *SPL5*) generally become up regulated during flower development (Cardon et al., 1999; Schmid et al., 2005).

Until now, little data is available concerning the functions of the SBP-box genes in *Arabidopsis*. Only two *Arabidopsis SPL* mutants have been described in the literature. *Spl8,* which affects anther and ovule development (Unte et al., 2003) and *spl14,* which participates in sensitivity to the fungal toxin fumonisine B1 and in the development of the leaf shape (Stone et al., 2005). In addition, transgenic plants overexpressing *SPL3* were described as early flowering (Cardon et al., 1999).

Besides these *Arabidopsis SPL* genes, few other SBP-box gene mutants have been identified. In *Zea mays*, mutations in the gene *LIGULESS* (*LG1*) resulted in plants lacking the ligule at the boundary between blade and sheath of the leaf (Moreno et al., 1997). A single amino-acid exchange in the SBP-box gene, *teosinte glume architecture* (*tga1*) from maize was shown to be responsible for differences in the architecture of the maize compared to the teosinte female inflorescence (ears) (Wang et al., 2005). Interestingly these differences are believed to be essential on the domestication of maize. Furthermore, in *Chlamydomonas reinhardtii* the SBP-domain protein *CRR1* has been shown to regulate copper homeostasis (Kropat et al., 2005).

Recently, Birkenbihl and co-workers were able to show that the palindromic tetranucleotide GTAC is essential for DNA binding by SPL1, SPL3 and SPL8. In addition Birkenbihl et al. showed that the putative NLS, harboured by the SBP-domain, is functional (Birkenbihl et al., 2005). Yamasaki and co-workers resolved the structure of the DNA-binding domains of SPL4 and SPL7, by using heteronuclear NMR spectroscopy. Their results revealed a novel type of zinc-binding structure containing two zinc-binding sites by eight of the ten conserved Cys or His residues (Yamasaki et al., 2004, 2006).

1.3 *SPL* genes and their regulation by MicroRNAs

MicroRNAs (miRNAs) are small (~21nt) non-protein-coding RNAs that pair to nearly complementary sites within their target mRNAs, triggering either translational repression or transcript degradation. In *Arabidopsis thaliana*, 92 loci have been identified encoding 27 miRNAs (Válóczi et al., 2006). Recent work has shown that the posttranscriptional gene regulation by miRNAs plays an important role in plant growth and development. Interestingly, eleven of the 17 members of the *SPL* genes in *Arabidopsis* have been predicted to be targets of the miRNA156 and the very similar miRNA157 (Rhoades et al., 2002). The miRNA recognition site was found to be in the last exon, either in the 3' UTR, as for *SPL3*, *SPL4* and *SPL5* or in the coding region, as for the other eight genes. Northern blot analysis showed that both, miRNA156 and miRNA157 are expressed predominantly in seedling stage. Later in development miRNA156 is expressed in flowers and siliques (Reinhart et al., 2002). Recently, Válóczi and co-workers used *in situ* hybridization to show an accumulation of the miRNA156 in young leaves, ovules and meristematic tissues (Válóczi et al., 2006).

MiRNA156 and miRNA157 can potentially be encoded by multiple loci in the genome. Constitutive overexpression of at least one of these, *MIRNA156b,* has been shown to cause a moderate delay in flowering of six days under long day conditions. In addition, *MIRNA156b* overexpressors initiate leaves faster than wild type. Furthermore, increased levels of miRNA156 cause a severe loss of apical dominance (Schwab et al., 2005).

Interestingly, global expression profiling revealed that transcript levels of eleven of the 15 *SPL* genes present on the ATH1 micro-array were substantially reduced in the young inflorescence apex. All of the eleven genes carried the predicted miRNA156 target site. The remaining genes without miRNA156 target sites remained largely unaffected (Schwab et al., 2005). In this context it is noteworthy that the strongest reduction in expression upon overexpression of the miRNA156 was observed for *SPL5*, *SPL15* and *SPL10*.

Based on the above summarized data on the expression profile of the *SPL* genes and the description of the effects of the overexpression of the miRNA156, a role for the SBP-box genes in floral transition, apical dominance and in the control of the plastochron seems to be likely.

1.4 The Shoot Apical Meristem and the Formation of Lateral Organs

Continuous growth of all aerial organs after germination is maintained through self renewing stem cells located at the shoot apical meristem (SAM). The SAM of mature plants can be subdivided into three domains: the central zone, the peripheral zone and the rib zone. In the central zone stem cells divide slowly and produce two types of daughter cells. Those daughter cells that stay in the center remain undifferentiated stem cells whereas the second type of daughter cells is displaced outward towards the peripheral and the rib zones (Fletcher, 2002; Williams and Fletcher, 2005). Cells in these two zones divide more rapidly and provide the founder cells for the formation of lateral organs and the stem, respectively. Remarkably, despite the fact that daughter cells are deposited continuously to initiate lateral organs, the number of stem cells in the central zone remains constant. This indicates a precise balance between the formation of new stem cells and the recruitment of daughter cells for the formation of lateral organs (Traas and Doonan, 2001). In *Arabidopsis*, a stem cell population even exists after the transition from vegetative to reproductive growth, allowing the inflorescence SAM to produce flowers in an indeterminate fashion.

Arabidopsis SAM maintenance involves a spatial negative feedback loop between the small signaling peptide CLAVATA3 (CLV3) and the homeodomain protein WUSCHEL (WUS), (Lenhard and Laux, 2003). *WUS* is expressed in a small group of cells underneath the stem cell region termed the organizing center (OC) and is essential for the maintenance of the overlying stem cell reservoir (Mayer et al., 1998). The stem cells signal back to the OC via the CLV signaling pathway (Schoof et al., 2000). CLV3, which is produced by the stem cells, binds to the CLV1-CLV2 receptor complex in the underlying cells and thereby represses the expression of *WUS*. If the number of stem cells

increases, more CLV3 peptide is produced which in turn causes down regulation of *WUS*. Fewer *WUS* expression reduces the number of stem cells and consequently results in a reduction of *CLV3* expression. This negative feedback mechanism guarantees a strict stem cell homeostasis (Carles and Fletcher, 2003).

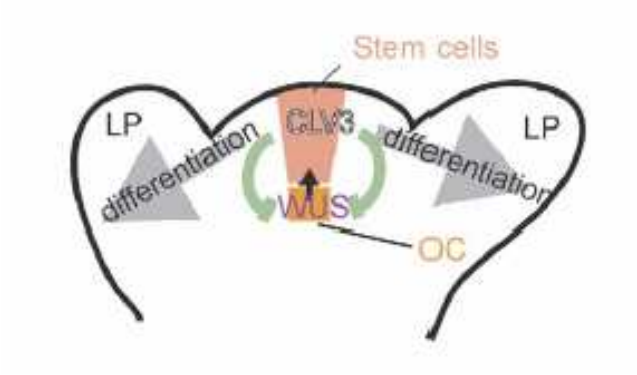

Figure 1.2.: Control of stem cells at the SAM
A stem cell pool is located above the organizing center which expresses the WUSCHEL (WUS) transcription factor. WUS controls transcription of CLAVATA3 (CLV3) expressed in the stem cells overlying the OC. CLV3 represses the expression of WUS. Stem cell daughters that leave the stem cell domain differentiate and form leaf primordia (LP). (Picture taken from Scheres, 2005).

Besides the histological subdivision of the SAM in central, peripheral and rib zone, the cells in the SAM can also be grouped in cell layers according to their clonal relationships: the epidermal (L1), the subepidermal (L2) and the underlying layers (L3).
The cells of the L1 and L2 layers are characterized by a predominantly anticlinal division plane and are ultimately incorporated into the epidermal and subepidermal layers of lateral organs.
Cells belonging to the L3 layer divide in a more complex manner and form the inner tissue of lateral organs and the stem pith.

The balance between the indeterminacy of the SAM and the determinate growth of lateral organs has to be maintained. Transcription factors of the KNOTTED1-like homeobox family (KNOX) and plant hormones are known to be key players in this respect (Kepinski et al, 2006).
The gene *SHOOT MERISTEMLESS* (*STM*) in *Arabidopsis* encodes a *Knotted1*-like homeodomain-containing protein and is required for the initiation and maintenance of the SAM (Long et al, 1996). Loss of *STM* results in failure to maintain a SAM (Endrizzi et

al., 1996). *STM* is expressed throughout the shoot apical meristem but down regulated in incipient leaf primordia (Barton and Poethig, 1993; Long et al., 1996; Endrizzi et al., 1996). The exclusion of *KNOX* expression from leaves is important for leaf development and marks a change in cell fate from meristem to leaf (Smith et al., 1992). KNOX proteins, as members of the TALE superclass of homeodomain proteins can interact with a second group of TALE proteins, the BEL1 homeodomain (BLH) family (Bellaoui et al., 2001). It has recently been shown that *STM* is targeted into the nucleus as a heterodimer with different BEL1-like (BLH) homeodomain transcription factors expressed in discrete sub-domains of the SAM (Cole et al., 2006), indicating that different combinations of STM/BLH transcription factors may regulate different downstream events.

An important KNOX action is to enhance the level of the plant hormone cytokinin (CK) and to suppress the levels of gibberellin (GA) in the SAM (Jasinski et al., 2005). The control of CK/GA homeostasis by KNOX contributes to the undifferentiated state of the SAM.

During development, lateral organs like leaves, flowers and floral organs emerge in a highly regular manner, a phenomenon referred to as phyllotaxis. Leaves for instance tend to be formed at a certain minimal distance from each other. In the spiral phyllotaxis of *Arabidopsis* this certain distance appears to be 137.5°. In a series of experiments, it has been shown that auxin (indole-3-acetic acid, IAA) is an essential activator of primordia formation. Reinhardt and co-workers proposed a model in which auxin is transported acropetally towards the SAM where it is then redirected to the leaf primordia (Reinhardt et al., 2003). According to this model, new primordia function as sinks and as a result the surrounding area is depleted of auxin. Only at a certain minimal distance from the last two initiated primordia (P1 and P2) auxin can accumulate and induce a new primordium (incipient primordium, I1) which, in the course of the plastochron, grows out and becomes a sink itself (Smith et al., 2006; Jönsson, 2006; de Reuille, 2006). Mediated by the actively transported hormone auxin the interaction between existing and incipient primordia in a growing apex results in highly regular phyllotactic patterns.

Polar auxin transport is mediated by cellular efflux and influx carriers. AUXIN RESISTANT1 (AUX1), the founding member of the auxin influx carriers (AUX/LAX family) as well as PIN1, a member of the auxin efflux carrier family, are both expressed

in the shoot meristem of *Arabidopsis* (Reinhardt, 2005). *AUX1* is expressed in the epidermal L1 layer of the meristem leading to an accumulation of auxin in L1. *PIN1* is expressed in the same cells but localized at the upper side, pointing towards the meristem center. The positioning of both AUX1 and PIN1 suggests a transport of auxin upwards into the meristem through the L1 layer (Reinhardt et al., 2003).

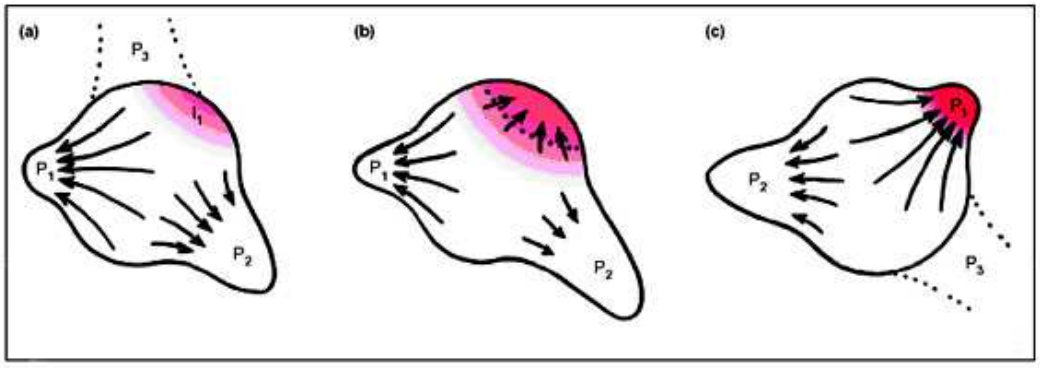

Figure 1.3.: Positioning and outgrowth of lateral organs during the phyllotactic cycle.
(a) As a result of the sink function of the primordia P1 and P2, auxin (red) can accumulate only at certain minimal distance from P1 and P2, which corresponds to the site of incipient organ (I_1) formation. **(b)** At a certain auxin concentration in I1, PIN1 becomes induced and begins to actively accumulate auxin at the incipient organ site. The sink activity of P1 and P2 decreases at the same time. **(c)** The auxin concentration now peaks at P1 (former I_1!) and leads to the outgrowth of a new organ. Black arrows indicate polar auxin transport. For clarity, auxin concentration (red) is only depicted at I_1. (Picture taken from Reinhardt, 2005).

Taken together, first auxin is distributed uniformly throughout the meristem. It then accumulates in primordia and at the same time is depleted from their vicinity. Accumulation of auxin is possible only at a certain minimal distance from the pre-existing primordium – beyond the 'reach' of it. At this point *PIN1* expression is induced and active auxin accumulation is initiated. The actively generated auxin maximum leads to the delimitation of the incipient primordium and auxin depletion from adjacent cells.
One of the most important aspects of the model described above is the polar distribution of auxin mediated by the polar localization of PIN1. Muday and co-workers showed that PIN1 is continuously endocytosed to endosomal compartments, and recycled back to the plasmalemma, thus providing a dynamic cellular mechanism that enables rapid changes in PIN1 localization (Muday et al., 2003). An important factor in PIN1 localization is the protein kinase PINOID (PID) (Friml et al., 2004). PID decides at which end of the cell PIN1 will accumulate.

Having specified the position at which leaves initiate, auxin also plays a role in tissue pattern formation, i.e. the development of vascular tissue and the dorsoventrality of the leaf (Kepinski, 2006).

Auxin inducible genes are activated and deactivated by complex interactions between two families of transcription factors, auxin response factors (ARFs) and Aux/IAAs (Dreher et al., 2006). *Aux/IAA* genes encode small nuclear proteins that have a common four domain structure. Through their conserved domains III and IV, Aux/IAA proteins can interact with each other and with similar domains of auxin response factors (Tiwari et al., 2004). Under low-auxin conditions, Aux/IAA proteins are able to repress the activity of ARF transcription factors. An increased level of auxin triggers the degradation of Aux/IAA proteins which in turn derepresses ARF activity and finally results in numerous auxin-mediated transcriptional changes (Dreher et al., 2006).

1.5 The Shoot Apical Meristem and the Floral Transition

Flower development can be divided into four steps. First, the plant switches from vegetative development to reproductive development in response to environmental and endogenous cues. Subsequently signals from various flowering time pathways lead to floral identity of meristems. In a third step the meristem identity genes activate the floral organ identity genes in discrete areas of the flower primordium. Fourth, various cell types and tissues that constitute the floral organs are specified by genes downstream of the floral identity genes.

Four major pathways are known that promote the switch from vegetative growth to reproductive growth within the life cycle of *Arabidopsis*: long-day photoperiod, gibberellin (GA), autonomous and vernalization.

Many genes involved in the photoperiod pathway encode proteins for light perception, e.g. phytochromes and cryptochromes or components of the circadian clock (e.g. *GIGANTEA* and *ELF3*) (Koorneef et al., 1991, Reeves and Coupland, 2000). Both the light and clock components ultimately lead to the activation of the nuclear protein *CONSTANS* (*CO*) (Guo et al., 1998; Suarez-Lopez et al., 2001).

Mutants involved in the gibberellin pathway (e.g. *ga1*) exhibit dramatic delays in flowering when grown under short day conditions but not under long day (Wilson et al., 1992; Blazquez et al., 1998). This suggests that the plant hormone gibberellic acid acts as an important stimulator of flowering in non inductive short day conditions. One target of the GA signal is *LEAFY* because *LFY* promoter activity is reduced in a *ga1*-3 mutant and increased by exogenous GA application (Blazquez et al., 1998; Boss et al., 2004).

Genes involved in the autonomous pathway (e.g. *FLD*, *FCA*) function to control flowering independent of environmental signals such as day length in reducing *FLOWERING LOCUS C* (*FLC*) mRNA accumulation (Michaels and Amasino, 2001; Schomburg et al., 2001). *FLC* itself is a MADS-box transcription factor that represses flowering through the repression of the floral pathway integrators *FLOWERING LOCUS T* (*FT*), *LFY* and *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS* (*SOC1*) (Boss et al., 2004).

The fourth major pathway is the vernalization pathway. Vernalization, the process that occurs in plants as they overwinter for many weeks in low temperatures, strongly downregulates *FLC* and so accelerates flowering (Sheldon et al., 2000; Ratcliffe et al., 2001; Boss et al., 2004).

The flowering time genes themselves control two groups of meristem identity genes, the shoot and the floral meristem identity genes.

The shoot meristem identity genes such as *TERMINAL FLOWER* (*TFL1)* specify the inflorescence apical meristem (IM) as indeterminate and nonfloral (Ruiz-García et al., 1997; Jack, 2004). In contrast, floral meristems (FMs) ultimately terminate in the formation of floral organs namely sepals, petals, stamen and carpels rather than leaves and shoots. The different identity of FM and IM is conferred by floral meristem identity genes such as *LEAFY* (*LFY*) and *APETALA1* (*AP1*) which are transcribed in initiating floral meristem primordia but not in the IM (Blázquez and Weigel, 2000). Both *LFY* and *AP1* encode transcription factors. *AP1* is a member of the MADS family whereas *LFY* encodes a protein without strong similarity to any other *Arabidopsis* proteins. Ectopic expression of *LFY* or *AP1* converts the inflorescence apical meristem into a flower.

Conversely in *lfy* and *ap1* mutants, flowers are either replaced by vegetative shoots or have vegetative characteristics (Weigel et al., 1992).
Other secondary floral meristem identity genes are *CAULIFLOWER*, *FRUITFULL* and *AP2* (Jack, 2004).
One of the important functions of the meristem identity genes is to activate the ABC floral organ identity genes (Bowman et al., 1991, Lohmann, 2002).
The existence of floral homeotic mutants that did not affect organ number or organ position, just organ identity, demonstrated that the regulation of organ formation is separable from the regulation of organ identity.
The mutants were defined as class A, class B and class C genes. The mutant phenotypes indicated that class A genes are required for the development of sepals and petals (e.g. *AP2*), class B genes are required for the development of petals and stamen (e.g. *AP3*, *PI*) and class C genes are required for the development of stamen and carpels (e.g. *AG*).

Since Cardon and co-workers were able to show that overexpression of *SPL3* causes early flowering, an involvement of the Arabidopsis *SPL* genes in floral development has been discussed (Cardon et al., 1997). Further hints came from an experiment which used global transcriptional profiling to investigate the response to photoperiodic induction at the shoot apex (Schmid et al., 2003). Remarkably, *SPL3*, *SPL4* and *SPL5* were up-regulated very strong upon photoperiodic induction. *SPL2*, *SPL6*, *SPL9*, *SPL10*, *SPL11*, *SPL13* and *SPL15* showed a similar but less strong upregulation. All ten *SPL* genes that responded upon induction are targets of the miRNAs156/157. Interestingly, *SPL3*, *SPL4* and *SPL5*, the three genes with the strongest reaction are distinguished from the rest by the presence of the miRNA target motive in the 3' UTR rather than in the coding sequence.

1.6 Project objective

The work described in this thesis aims at better understanding of the biological role of SBP-box genes in *Arabidopsis thaliana*. In particular, two main questions were tried to be answered in the course of this study:

When the work of this thesis started, *SPL8* was the only known *SPL* gene with a mutant phenotype indicating a defined role in development. Thus, the first question concerned the target genes of *SPL8*. As mentioned before, mutation of *SPL8* caused a strong reduction in fertility, mainly as a consequence of abnormal cell differentiation in the developing anthers. In an attempt to gain insight in the developmental pathways *SPL8* is acting in, a global expression profiling with help of the micro-array technology should help to identify putative target genes of SPL8. Subsequently, semi-quantitative RT-PCR should verify the micro-chip data. In addition, analysis of the putative target gene promoters with respect to the SBP-domain DNA binding motive GTAC, should facilitate the search for direct targets. The combined data would allow placing *SPL8* in a regulatory network responsible for anther development in *Arabidopsis*.

The second part of this project aims to elucidate the biological function of the other *SPL* genes. Reverse genetics as a powerful tool for functional genomics should help drawing conclusions from phenotypic alterations to possible gene function. *SPL* gene mutants should be identified in large T-DNA mutagenized *Arabidopsis* populations. The characterization of morphological changes displayed by the mutants under a wide range of environmental conditions in combination with information about mRNA expression patterns provided by northern blot analysis (Cardon et al., 1997, 1999) and global expression profiling (Schmid et al., 2005) should help to shed light on the molecular function of the SBP-box transcription factor family.

In a longer perspective, the results of this study should help to give an answer on the questions whether all 17 members of the *SPL* genes in *Arabidopsis* are to be placed in the same functional network, whether certain *SPL* genes can be subgrouped according to their function or whether different *SPL* genes have completely different functions.

Furthermore, the results gained from this work should help to answer the question whether the *SPL* gene functions can be generalized beyond the model plant *Arabidopsis thaliana* onto other plant species.

2 Material and Methods

2.1 Material

2.1.1 Antibiotics

Antibiotics	Stock Conc. (mg/ml)	Solvent	Final Conc. (mg/l)	
			E .coli	***A .tumefaciens***
Gentamycin	10	H_2O	10	25
Rifampycin	50	MetOH	100	100
Spectinomycin	100	H_2O	100	100
Kanamycin	50	H_2O	50	50

Table 2.1.: Antibiotics used in this study.

2.1.2 Bacteria strains

E.Coli* strain*:

DH5α (Invitrogen, USA)

***Agrobacterium tumefaciens* strains:**

GV3101 (pMP90)

GV3101 (pMP90RK)

2.1.3 Primers for PCR based amplification methods

All Primers used were purchased from Invitrogen (Netherlands). Primer sequences are listed in the appendix.

2.1.4 Plant material and plant growth conditions

All plants grown on soil (containing a mixture of substrate and vermiculite, 3:1) were kept under controlled environmental conditions (22°C, 50% RH) and 150µE*m-2*s-1 light (fluorescent Sylvania F72T12 cool-white [75%] and incandescent Sylvania 100-W lamps [25%]) either under long day conditions (16 hours light followed by 8 hours darkness) or under short day conditions (8 hours light followed by 16 hours darkness). Before sowing, seeds were kept on moist paper at 4 °C in dark for 4 to5 days to break dormancy and synchronize germination.

For some experiments, seeds were surface sterilized, plated on germination medium and cold treated for 4 days before placing them either vertically or horizontally under long day conditions.

2.1.5 Media, Buffers and Solutions

2.1.5.1 General buffers and solutions

TE (Tris/EDTA)

10mM Tris/HCL (pH 8.0)

1mM EDTA (pH 8.0)

Tris/HCL (1M)

Tris-Base	121g
H_2O	1000ml

EDTA (0.5M, pH 8.0)

EDTA	186.1g
H_2O	1000ml

Sodium phosphate buffer (0.2M), pH 7.0

Solution I: 0.2M monobasic phosphate

2.4g NaH_2PO_4 (per 100ml)

Solution II: 0.2M dibasic phosphate

3.56g Na_2HPO_4 (per 100ml)

To obtain 0.2M PO_4-buffer with a pH 7.0 19.5ml of solution I was mixed with 30.5ml of solution II.

Ethidium Bromide stock (10mg/ml)

Ethidium bromide	0.2g
H_2O	20ml

Store at 4°C in the dark.

DNA gel loading buffer (6x)

Bromphenol blue	0.25%
Xylen cyanol FF	0.25%
Glycerol	30%

GUS histochemical Buffer

NaPO4	0.2 M
$K_3Fe(CN)_6$	0.05 M
$K_4Fe(CN)_6$	0.05 M
EDTA	0.5 M
Triton X-100	10%
H_2O	

Store at 4 °C.

GUS staining Buffer (10ml)

X-Gluc stock (50mg/ml)	0.12ml
GUS histochem. Buffer	8ml
Methanol	2ml

2.1.5.2 Buffers for bacteria DNA manipulation

TELT (DNA extraction buffer)

Tris/HCL pH7.5	50mM
EDTA	62.5mM
LiCL	2.5M
Triton X-100	0.4%, autoclave

Lysozyme solution

Lysozyme	10mg/ml
Tris/HCL pH 7.5	10mM
EDTA	0.1mM

2.1.5.3 Buffers for plant DNA manipulation

Plant genomic DNA extraction buffers (Edwards Buffer)

Tris/CL pH7.5	200mM
NaCL	250mM
EDTA	25mM
SDS	0.5%

Plant genomic DNA extraction buffers (CTAB Buffer)

Tris/HCL, pH8.0	100mM
NaCl	1.4M
EDTA	20mM
CTAB	2%

2.1.6 Media for bacteria and plant growth

YEB Medium

Beef extract	5g/l
Yeast extract	1g/l
Peptone	1g/l
Sucrose	5g/l, pH 7.5
1M MgSO4	2ml/l after autoclaving

For solid medium add 15g/l Agar prior autoclaving.

LB (Lauria Bertani)-Medium

Tryptone/peptone	1%
Yeast extract	0.5%
NaCl	0.5%

For solid medium, 1.5-2% Agar was added to the above medium.

After autoclaving and cooling down to 55°C, antibiotics was added.

Infiltration Medium

1/2 MS salts (micro and macro)	2.205g/l
1/2 x B5 vitamins	50µl/l
Sucrose	50g/l
Surfactant SILWET L-77	0.005%
pH 5.7 (KOH)	

Germination Medium

1 x MS salts	4.4g/l
Agar	8g/l
pH 5.7 (KOH)	

2.1.7 Enzymes

Restrictions enzymes were purchased from New England Biolab (NEB) and Roche (Mannheim). T4 DNA ligase was purchased from Roche (Mannheim). Reverse transcriptase Supersript II and Superscript III were purchased from Invitrogen (USA). One-step RT-PCR was conducted using the on-step RT-PCR kit from Qiagen.
All enzymatic reactions were done according to the manufactures manual.

2.1.8 Chemicals

Chemicals were purchased from the following companies: BioRad (USA), Clontech (Germany), Difco Lab (USA), Duchefa (Netherlands), Invitrogen (USA), MBI Fermantas (Germany), Merck (Germany), Pharmacia (USA), Promega (Germany), Roche (Germany) and Sigma (Germany). Radioisotop (α^{32}P-dCTP) was purchased from Hartmann Analytics (Braunschweig). All antibiotics were purchased from Duchefa (Netherlands). For DNA purification and PCR product purification the NucleoSpin® Columns (Macherey-Nagel) was used.

2.2 Methods

2.2.1 Sequencing

All sequencing reactions were done by the ADIS core facility at the MPIZ in Cologne.

2.2.2 Genomic DNA extraction from plant material

In order to extract plant genomic DNA, a method from Edwards et al (1991) was adapted. Two to three young leaves were grinded in 400µl Edwards buffer. The mixture was vortexed and subsequently centrifuged for 3 minutes at full speed. 300µl of the supernatant was transferred into a new tube and 300µl isopropanol added, followed by a centrifugation step of 5 minutes at full speed. The precipitated DNA was washed with 70% ethanol and tried at the air. 200µl Tris pH 8.0 was used to dissolve the DNA pellet.

2.2.3 Isolation of plasmid DNA

For plasmid DNA isolation the NucleaoSpin® Kit (Macherey-Nagel) was used according to the manufacture's protocol.

2.2.4 Standard PCR reaction

All PCR reactions were done on a MJ Research PTC-200 (Biozyme) thermo-cycler. For standard reactions (genotyping), PEQ Gold *TAQ*-DNA Polymerase from PeqLab was used. For high accuracy PCR reactions (e.g. Cloning), *PFU*-Polymerase was purchased from PeqLab or Stratagene.
A standard PCR reaction was done as follows:

2mM	dNTPs	5µl
10pmol/ul	Primer 1	2.5µl
10pmol/ul	Primer 2	2.5µl
10x	PCR Puffer	5µl
	DNA Template	1µl
	H_2O	28µl
	DNA Polymerase	1µl
		50µl

Annealing Temperature	55 °C – 65 °C
Elongation Time	1minute/1kb
Number of cycles	28-35

2.2.5 Total RNA extraction from plant material

Total RNA was extracted using the Qiagen *RNeasy* Kit according to the manufacturer's protocol. On column DNA digestion was performed during the RNA extraction procedure using On-column DNAse from Qiagen according to the manufacturer's recommendation.

2.2.6 Semi-quantitative RT-PCR reaction

Semi-quantitative RT-PCR was done either in one step using the One-step RT-PCR kit (Qiagen) or in two steps using Superscript II/III (Invitrogen) according to the manufacturer's manual.

2.2.7 Identification of SPL8 target genes

A 1376 bp fragment, containing *alcA35S::GFP-ER* and the 35S terminator was cut out of pGreen 0129AlcR alcAGFP-ER + alcA35S (provided by Sabine Zachgo, MPIZ Cologne) using HindIII. The entire fragment was cloned into the HindIII site of the binary vector pBAR-A. In a next step the GFP fragment was cut out using SmaI. Instead, *SPL8*, fused to a *VP16* activation-domain via a XhoI site on the N-terminus was cloned into this SmaI site.

The construct (StK002) was transformed into Col-0 plants being transgenic for *AlcR* (Roslan et al., 2001) by means of Agrobacterium infiltration (Errampalli et al., 1991).

The induction experiment was done as follows: three independent lines homozygous for *AlcR AlcA35S::VP16:SPL8* and one control line containing only *AlcR* were grown for 17 days under long day conditions in pots of nine plants each. After 17 days these plants were placed next to a beaker containing 10% ethanol and covered immediately for the desired amount of time. Control plants were covered together with a beaker containing water. Six to seven leaves were shock frozen in liquid nitrogen before and four as well as eight hours after induction respectively. The extracted total RNA was stored at –80 °C before processing. RNA extraction was done using the *RNeasy Kit* from Qiagen.

The preparation of the total RNA was done according to Affymetrix Probe Synthesis Guidelines, (Markus Schmid and Jan Lohmann, MPI Tübingen; www.weigelworld.org/resources/microarray/AtGenExpress/AtGE_probe_synthesis.pdf). Probes were hybridized onto the Affymetrix ATH1 array according to the manufacturer's recommendations. Expression estimates were calculated using gcRMA implemented in R using default settings.

2.2.8 Mutant Screen

All 17 members of the *SPL* gene family were screened for available T-DNA insertion lines using the *SIGnAL "T-DNA Express" Arabidopsis Gene Mapping Tool* (http://signal.salk.edu/cgi-bin/tdnaexpress).
PCR was used in order to test whether the T-DNA inserted at the predicted insertion site. All T-DNA insertions were confirmed by PCR using one primer specific to the left border of the inserted T-DNA and one primer specific for the flanking genomic sequence. In addition two primers flanking the putative insertion site were used. RT-PCR was used subsequently in order to test the transcript level of the respective gene.

2.2.9 *pSPL15:GUS* reporter gene construct

pSPL15::GUS:SPL15 was constructed by subcloning a 2793bp genomic fragment of *SPL15* (At3g57920) beginning at an artificial EcoRI site 1260bp upstream of the ATG start codon and extending to an artificial EcoRI site 132bp downstream of the stop codon into the EcoRI sites of the binary vector pGJ2148 (Guido Jach, MPIZ Cologne). An 1832bp GUS fragment was subsequently cloned in frame +73bp from the ATG start codon using ScaI.

2.2.10 *SPL15:YFP* reporter construct

35S::SPL15:YFP was constructed by subcloning a *SPL15* cDNA fragment of 1062bp into pDONR 201 (Gateway, Invitrogen). In order to PCR-amplify the cDNA fragment, the following primer's were used: forward 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGGAGTTGTTAATGTGTTCGGG-3'; reverse 5'-GGGGACCACTTTGTACAAGAAAGCTGGGTCAAGAGACCAATTGAAATGTTG AGG-3'. The fragment was subsequently cloned into pEXSG YFP (Feys et al., 2005) according to the manufacturer's recommendation (Invitrogen). The construct was brought into Col-0 plants by means of *Agrobacterium* mediated transformation using the *Agrobacterium* strain GV3101 (pMP90RK). (The construct was kindly provided by Susanne Höhmann, MPIZ Cologne).

2.2.11 *SPL9:YFP* reporter construct

35S::SPL9:YFP was constructed by subcloning a SPL9 cDNA fragment of 1124bp into pDONR 201 (Gateway, Invitrogen). In order to PCR amplify the cDNAfragment, the following primer combination was used: forward 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGGAGATGGGTTCCAACTCGGG-3'; reverse 5'- GGGGACCACTTTGTACAAGAAAGCTGGGTCGAGAGACCAGTTGGTATGGTGAG-3'. The fragment was subsequently cloned into pEXSG YFP (Feys et al., 2005). According to the manufacturer's recommendation (Invitrogen). The construct was brought into Col-0 by means of *Agrobacterium* mediated transformation using the Agrobacterium strain GV3101 (pMP90RK). (The construct was kindly provided by Susanne Höhmann, MPIZ Cologne).

2.2.12 Genomic *SPL9* construct

In order to complement the *SPL9* loss-of-function phenotype a 5851bp genomic fragment of *SPL9* was amplified using a forward with an artificial SmaI site and a reverse primer with an artificial SacI restriction site. The fragment was subsequently cloned into the SmaI/SacI site of the vector pBAR-A. The construct was transformed into *spl9*-1 mutant plants by means of Agrobacterium mediated transformation.

2.2.13 GUS staining

GUS activity was detected in whole plants as follows. The plants were submerged in 10ml GUS staining buffer and subsequently put under vacuum for 10-15 minutes and incubated at 37 °C for 18 hours. The tissue was cleared by several changes of 70% ethanol until the chlorophyll was bleached. Depending on size, photographs of the plants were taken using Zeiss Axiophot or Leica PTC-200.

2.2.13.1 Preparation of Arabidopsis embryos for GUS staining

Siliques of different ages were removed form the plant. Using the binocular both valves were removed and the seeds were incubated in acetone for 30min on ice. Seeds were subsequently washed in 0.1M PO_4 buffer and stained over night at 37 °C using GUS staining buffer. At the next day seeds were washed in 0.1M PO_4 buffer and then cleared in chloral hydrate for at least 6 hours at 4 °C. GUS stained embryos were looked at using a microscope (Zeiss Axiophot).

3 Results

3.1 Identification of Downstream Target Genes of the Putative Transcription Factor SPL8

Unte et al. previously described that *SPL8* plays a major role in microsporogenesis and megasporogenesis within the anthers and ovules, respectively. In the absence of a functional *SPL8* gene *Arabidopsis* plants show severe defects in the proper initiation of microsporangium formation at defined positions within the anthers and for the regular entrance of spore mother cells into meiosis (Unte et al., 2003).

How exactly SPL8 is involved in the above-described processes, however, is still unclear. Since the SBP-domain protein SPL8 is assumed to act as a transcription factor it was of particular interest to uncover its target genes whose functions seem, at least in part, necessary for normal sporogenesis. To learn their functions would provide additional insight in this important developmental process.

In order to answer this question, a high throughput target gene search by means of microarray technology was conducted. The experiment was performed using "photolithographically" produced microarrys in which each gene is represented as a probe set with several oligonucleotides (25mer) (Affymetrix Gene Chips). The *Arabidopsis* ATH1 array (Affymetrix) that was used represents 22,810 such probe sets and assumed to cover approximately 90% of all *Arabidopsis* genes.

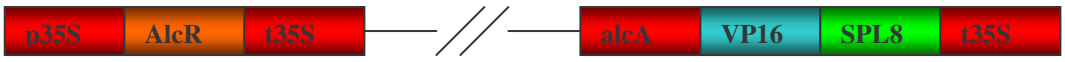

Figure 3.1.: The alcohol inducible *SPL8* system.
The alcohol receptor AlcR under the control of the strong, 35S promoter is expressed constitutively. Upon binding of ethanol the receptor protein induces the expression of genes downstream of the alcA promoter (here driving the expression of a *VP16:SPL8* fusion product).

Cardon et al. and Unte et al. have both shown that the expression domain of *SPL8* is very narrow in terms of spatial and temporal expression, i.e. very early during the flower development. Extracting enough mRNA, expressed during this stage, to compare differential gene expression in the wild type and the *spl8* mutant was therefore considered to be very difficult. To account for this problem, an ethanol inducible *SPL8* construct was used to ectopically overexpress *SPL8* (Figure 3.1.). This had the additional advantage that the moment at which the putative transcription factor SPL8 became activated and thus was able to induce expression of downstream target genes, could be controlled. The total RNA was extracted from rosette leaves of two week old seedlings, representing a developmental stage and tissue which *SPL8* is normally not expressed in. To be independent of any additional factors that might be necessary for normal SPL8 function in the flower but not present in leaves, the viral transcriptional activator domain VP16 (Triezenberg et al., 1988) had been fused to the N-terminal part of SPL8 (for more precise information, see part: "Materials and Methods").

After *Agrobacterium* mediated introduction of the transgene into plants constitutively overexpressing the alcohol receptor *AlcR*, many transgenic plants were obtained (Figure 3.2.). Three lines (2-4, 9-3 and 9-4) were selected for further experiments, each found to have high SPL8 expression upon ethanol induction compared to non-induced control plants as well as compared to alcohol induced AlcR plants (plants that only expressed the AlcR receptor but which were not transformed with the *alcA::VP16:SPL8* construct).

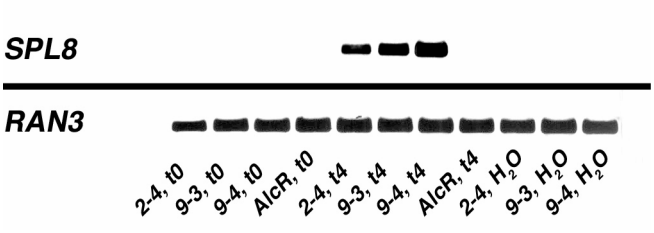

Figure 3.2.:
RT-PCR results of the three transgenic lines 2-4, 9-3 and 9-4. After ethanol vapor for four hours (t4). *SPL8* transcript level is high in all lines compared to non-induced plants (t0) and water treated control plants (H_2O). The AlcR line does not show any *SPL8* induction, even after four hours ethanol induction.

In order to hybridize the Affymetrix ATH1 arrays, total RNA from the *SPL8* inducible lines was extracted after four hours of ethanol induction. Control plants being transgenic

for the same construct were induced for four hours with water. As a further control, plants being transgenic for the alcohol receptor AlcR, but not for the *SPL8* construct were induced with ethanol for four hours.

Out of the approximately 24000 genes represented on the *Arabidopsis* ATH1 array, 28 top candidates for being SPL8 target genes were selected according to their strength of differential expression, comparing the *alcA::VP16:SPL8* plants with all possible controls. The cut off level was set at $2^{1.5}$ fold (Table 3.1.).

AGI-Code	Fold Increase (log2)	Annotation	RT-PCR result
At5g45960	39.5	GDSL-motif lipase	+
At5g55180	12.0	glycosyl hydrolase	+
At5g09440	9.9	phosphate-responsive protein	+
At1g56150	6.9	auxin-responsive family protein	NA
At3g53950	6.9	glyoxal oxidase-related	NA
At3g45060	6.8	high-affinity nitrate transporter	+
At1g44830	6.0	AP2 domain-containing transcription factor TINY	+
At3g26200	5.7	cytochrome P450 71B22	+
At4g08040	5.6	1-aminocyclopropane-1-carboxylate synthase	+
At3g57520	4.1	alkaline alpha galactosidase	-
At3g15270	3.6	squamosa promoter-binding protein-like 5 (SPL5)	+
At5g27920	3.3	F-box family protein	+
At1g16510	3.1	auxin-responsive family protein	+
At2g40330	2.7	Bet v I allergen family protein	-
At5g37580	2.7	tropomyosin-related low similarity to tropomyosin gene 1	-
At5g14230	2.7	ankyrin repeat family protein	+
At1g22030	2.5	expressed protein	NA
At3g57780	2.3	expressed protein	+
At1g17460	2.3	myb family transcription factor	-
At5g61460	2.3	structural maintenance of chromosomes (SMC) family protein	+
At3g50770	2.3	calmodulin-related protein	-
At4g19380	2.2	alcohol oxidase-related	NA
At3g63240	2.2	endonuclease/exonuclease/phosphatase family protein	+
At4g13100	2.1	zinc finger (C3HC4-type RING finger) family protein	+
At1g69760	2.0	expressed protein	-
At5g17760	2.0	AAA-type ATPase family protein	NA
At3g10570	1.9	cytochrome P450	+
At1g08210	1.9	aspartyl protease family protein	+

Table 3.1.: List of 28 SPL8 target genes.
The list shows the AGI-code and the annotation of the 28 selected putative SPL8 target genes as well as their fold increase in expression comparing the ethanol induced *VP16:SPL8* plants to the control plants. The fold increase is $\log_2$ transformed.
The expression of all genes was tried to be verified by means of RT-PCR. Genes marked with a "+" showed similar expression pattern in the RT-PCR as in the microarray experiment (i.e. high expression level in the alcohol induced sample compared to low expression level in the control sample). Genes marked with a "-"did not show the expected expression, for example appeared to have equal expression levels in the induced sample and the control sample. Genes marked with "NA"could not be amplified by RT-PCR because of technical reasons.

Changes in expression detected on the micro-array were confirmed by means of semi-quantitative RT-PCR (See Appendix, Figure AI).

For most candidate genes the results from the ATH1 Chip could be independently confirmed using the same total RNA samples that were used for the chip experiment. Most genes that showed strong differential expression on the array also showed significantly higher transcript levels after reverse transcription in the induced sample compared to the control sample. However, the RT-PCR also revealed some false positive genes. For example At3g57520, which showed strong differential expression on the chip ($2^{4.1}$ fold up-regulation in the induced sample compared to the control), seemed in fact to be induced by the AlcR protein rather than SPL8 because it showed strong induction in the alcohol induced AlcR control plants as well. Another example for a false positive signal turned out to be the gene At1g69760 which appeared to be up-regulated 2^2 fold in the chip experiment but when using RT-PCR on the same RNA seemed to be induced in all control samples as strong as in the *alcA::VP16:SPL8* plants.

Some genes, namely At1g56150, At3g53950, At4g19380, At5g17760 or At1g22030 could not successfully be amplified by means of RT-PCR. Therefore, the results gained by the Affymetrix ATH1 chip could not be verified for those.

However, the great majority of genes which showed strong differential expression in the micro-array experiment could be confirmed by semi-quantitative RT-PCR.

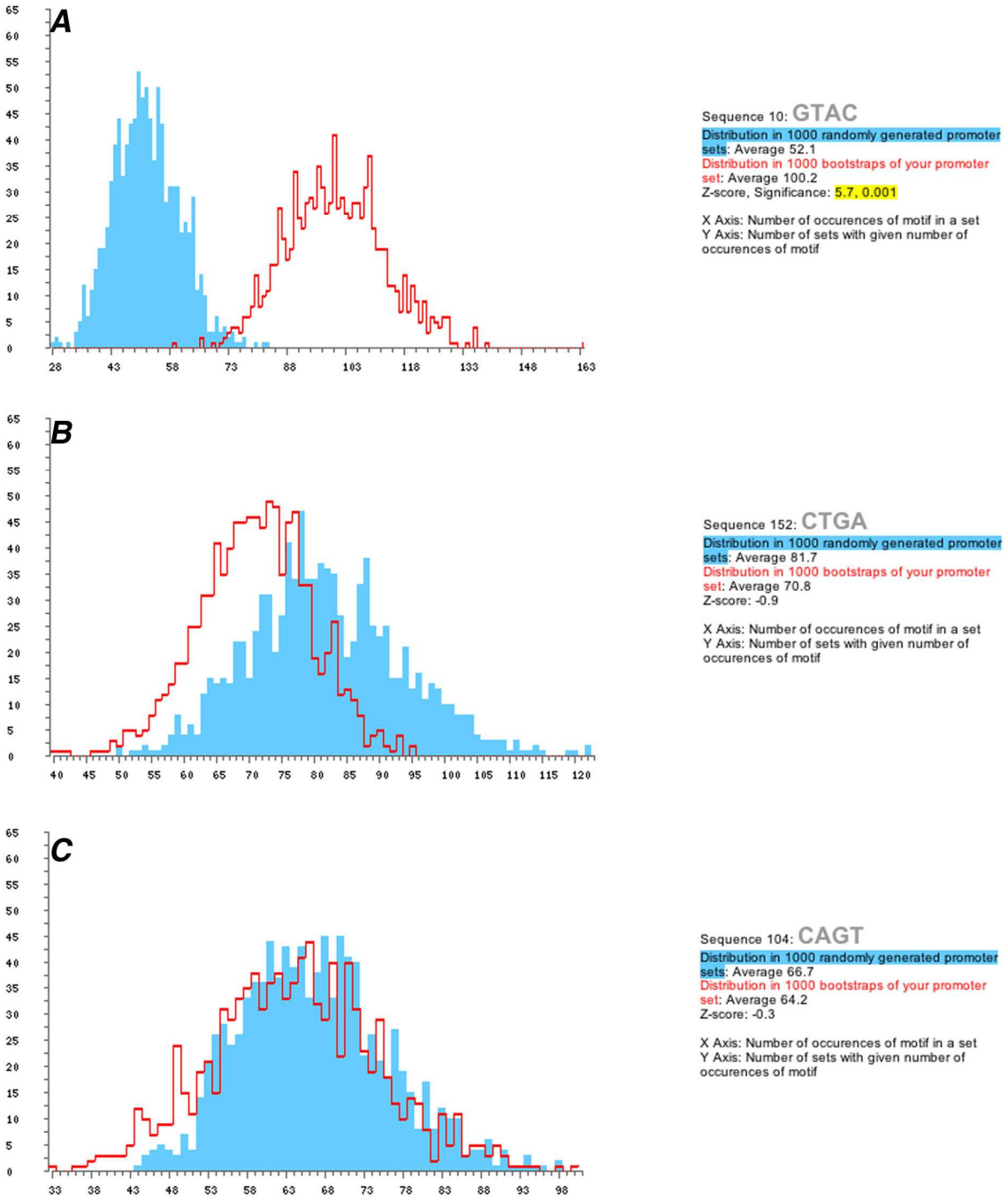

Figure 3.3.:
(A) The predicted DNA binding motif for the SBP-domain, the 4mer GTAC is statistically about 2 fold overrepresented in the 1-kb upstream sequences of the presumed translational starts of the 28 putative *SPL8* target genes compared to a random promoter set. Changing the sequence of the 4 nucleotides in CTGA **(B)** or CAGT **(C)** results in a distribution that does not show any significant difference between the random promoter set and the 28 selected genes.

Birkenbihl et al. previously showed that the DNA-binding domain of SPL8 specifically recognizes the 4mer GTAC (Birkenbihl et al., 2005). Following the expectation that putative target genes of SPL8 have the binding motif present in their promoter regions, a bootstrapping analysis was performed with 1000 control promoter sets (1-kb upstream sequence from the known or predicted translational start site), each of which contained 28 promoters from genes that were selected randomly from the *Arabidopsis* Gene Chip. In parallel the 28 putative SPL8 target genes also were bootstrapped to generate 1000 target gene sets (http://bbc.botany.utoronto.ca/ntools/cgi-bin/BAR_Promomer.cgi). Statistically, in a 1kb promoter the tetranucleotide GTAC was expected to appear 3.9 (without correcting for the GC content) times. In 28 promoters the number of occurrences was therefore expected to be 109 times.

As shown in figure 3.3. (A) the average number for the GTAC motif in the 1000 control promoter sets was 52.1, whereas the average number of the GTAC motif in the 28 *SPL8* target genes appeared to be 100.2. The P-value of less than 0.001 proved that the tetranucleotide GTAC was statistically significantly overrepresented in the 28 target genes as compared to the randomly selected gene cluster.

In fact the analysis revealed that the number of occurrences of the GTAC motif in the 28 target promoters matched almost the expected statistical value of 109 whereas the motif appeared to be 2 fold underrepresented in the whole genome promoter set.

As a comparison, the same statistical analysis was applied to two motifs (i.e. CTGA and CATG) containing the same nucleotides but at different positions than the true element GTAC. As shown in figure 3.3 (B) and (C) there was no significant difference in the frequency of neither of the two motifs between the control and the SPL8 target gene sets. Noticeable, the number of occurrences of the SPL8 binding motif GTAC in the random promoter set was underrepresented not only in comparison to the target gene promoter set but also in comparison to the two other random tetranucleotides tested. These data suggest that the GTAC motif occurs less frequent in the promoters of the genome than the two tested random motifs.

A further RT-PCR based test was performed on the selected candidate target genes. The expression level of the genes was tested in vegetative tissue and young flowers of wild-type plants, of *SPL8* overexpressing plants and of *spl8* mutant plants (Figure 3.4.). Since *SPL8* is naturally expressed mainly in very young flowers, putative target genes were expected to be expressed mainly in the flower samples as well. In addition, target genes that are activated by SPL8 should be expressed stronger in the *35S:SPL8* plants than in wild type and should be absent in *spl8* knockout plants. On the other hand, target genes that are repressed by SPL8 should to be expressed less in the *SPL8* overexpressor compared to the *spl8* mutant.

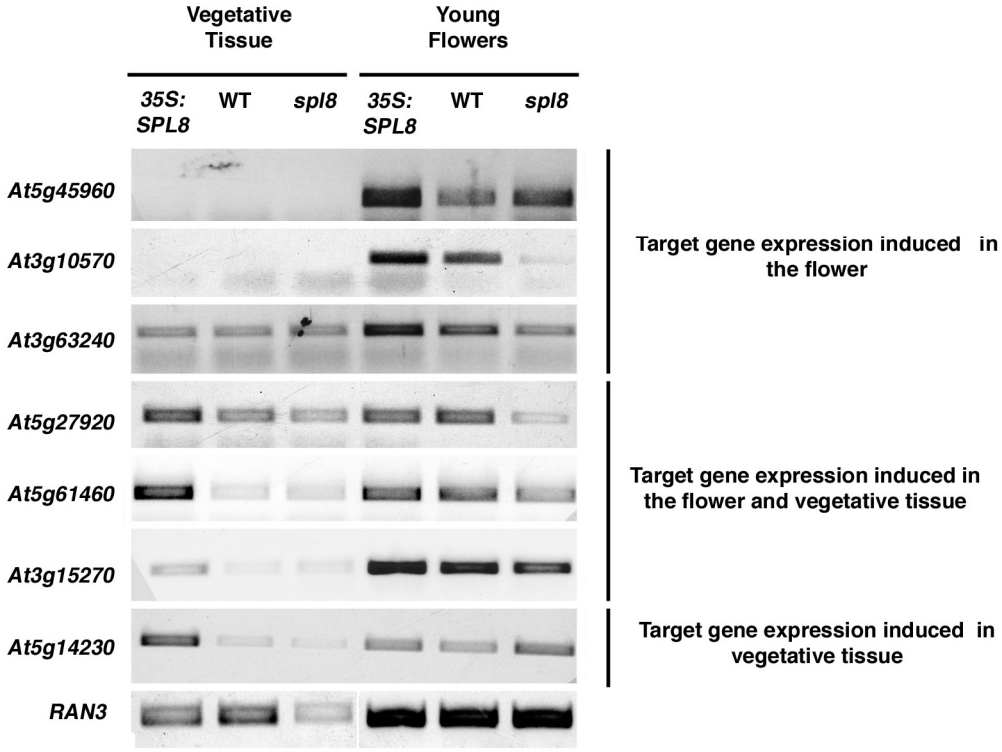

Figure 3.4.:
RT-PCR results of different putative SPL8 target genes in vegetative tissue and young flowers of *SPL8* overexpressor plants, wild type and *spl8* knock-out plants. The expression of At5g45960, At3g10570 and At3g63240 is induced by *SPL8* overexpression exclusively in young flowers. Gene expression of At5g27920, At5g61460 and At3g15270 however is induced by overexpression of *SPL8* in vegetative tissue as well as in flowers. The gene At5g14230 is induced in the vegetative tissue whereas expression in floral tissue is not changed upon overexpression of *SPL8*.

Of the putative target genes tested, At5g45960, At3g10570 and At3g63240 showed an expression pattern as it was expected for a target gene activated by SPL8. All genes were strongly upregulated exclusively in the flower of the *35S:SPL8* plants, but were almost absent in the *spl8* knock out. At5g27920, At5g61460 and At3g15270 showed a similar expression pattern but appeared to be upregulated in the vegetative tissue of the overexpressor as well.

At5g14230 responded to overexpression of SPL8 only in the vegetative tissue tested, but did not show any differential expression in the flower.

3.2 Insertional Knock-Out Mutants in the SBP-box Genes of *Arabidopsis thaliana*

As mentioned before, the transposon tagged *spl8* was the first mutant within the *Arabidopsis* SBP-box genes described (Unte et al., 2003). Mutation of *SPL8* resulted in aberrant pollen sack development.

In an attempt to gain more information about the function of the other 16 members of the *SPL* gene family in *Arabidopsis*, a reverse genetic approach was followed. Thereto, publicly available, electronic searchable databases for T-DNA mutagenized *Arabidopsis* populations (i.e. GABI-Kat, Cologne, Germany; SALK, La Jolla, USA and INRA, Versailles, France) for which T-DNA insertion sites had been determined were screened for insertions within the genomic regions of the *SPL* genes. Seeds of electronically identified T-DNA lines were ordered and subsequently checked by PCR whether the T-DNA was indeed inserted. In order to determine the exact position of the insert, the left border of the T-DNA was sequenced. Once identified as being complete, homozygous knock outs for the tagged gene, the plants were observed carefully for any phenotypical difference compared to wild-type plants of the respective ecotype on the hope to be able to draw conclusions from phenotypical alterations to possible gene functions.

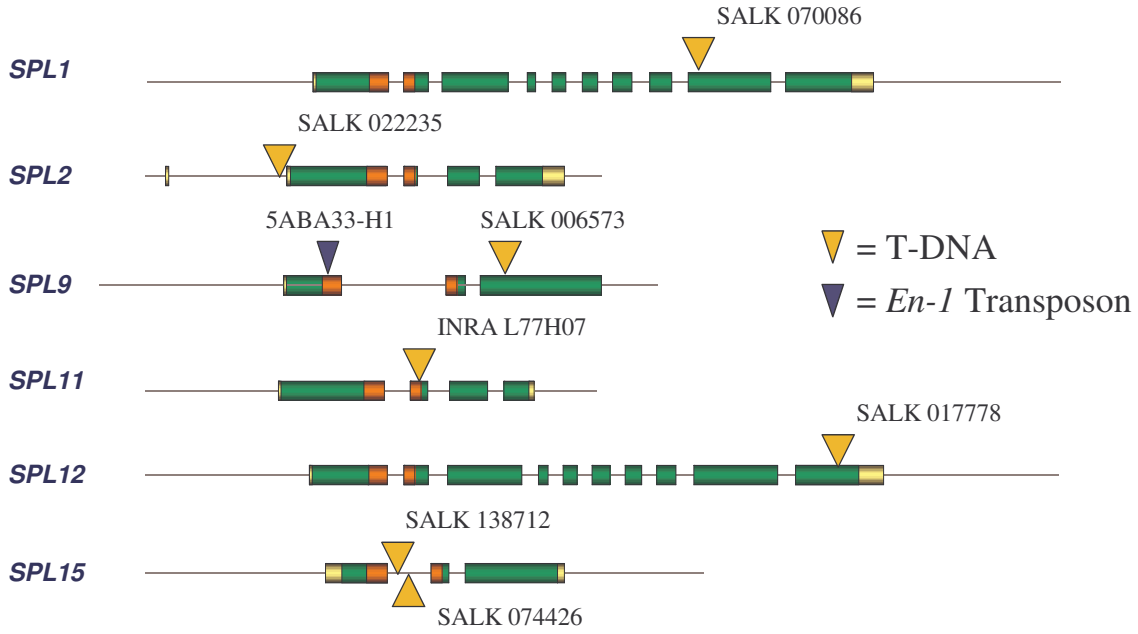

Figure 3.5.:
Schematic representation of different SPL genes and the respective T-DNA or transposon insertion site. Green boxes indicate Exons, red boxes indicate the SBP-box and yellow boxes indicate 5' and 3' UTRs.

Among all T-DNA collections that were screened, a total of six *SPL* genes were found to have one or more T-DNA insertion alleles such that transcription was abolished (Figure 3.5.).

In *SPL1* the SALK T-DNA 070086 is inserted 2610bp downstream of the presumed ATG start codon. For *SPL2* the SALK T-DNA 022235 is inserted 292bp upstream of the predicted ATG start codon. For *SPL9* a T-DNA insertion (06573) was found in the SALK collection that inserted 1369bp downstream of the presumed ATG start codon. U. Unte previously isolated a stable mutation 210bp downstream of this ATG caused by the insertion and subsequent excision of an En-1 transposon (U. Unte, PhD Thesis, 2001). In the INRA collection a T-DNA insertion (L77H07) was found 859bp downstream of the *SPL11* gene. For *SPL12* a T-DNA of the SALK collection (017778) was found to be inserted in the last exon, 3371bp downstream of the predicted ATG start codon. For *SPL15* two insertions were found within the SALK T-DNA collection both located in the first intron. The T-DNA 138712 inserted 422bp downstream of the presumed ATG and the T-DNA 074426 inserted 496bp downstream of the presumed ATG.

Primers upstream of the T-DNA insertion site were used for RT-PCR to confirm that the insertion indeed led to a transcriptional knock out (Figure 3.6.). In the cases of *SPL1* (SALK 070086), *SPL9* (SALK 06573) and *SPL12* (SALK 017778) in which the T-DNA inserted close to the 3' end of the coding region (see figure) a transcript upstream of the insertion site could be amplified. However, when using primers downstream of the insertion site the RT-PCR reaction failed, showing that no full transcript was present. Since no antibody was available for any of the respective genes it could not be entirely excluded whether those genes were not translated up to the T-DNA insertion and still gave rise to truncated but partially functional proteins.

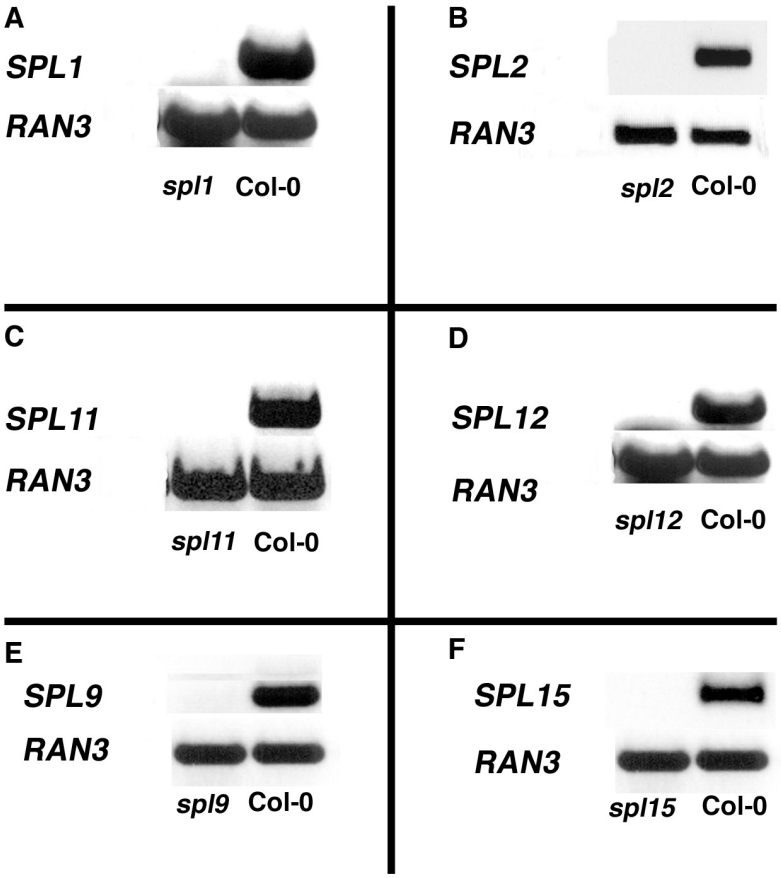

Figure 3.6.: RT-PCR results of all T-DNA insertion mutants.
The RT-PCR's showed no transcript of the respective genes in the mutants (left) compared to Col-0 wild type (right).

As was described by Cardon and co-workers the *SPL* genes can be grouped into sub-clades with respect to amino acid sequence similarity within the SPB box (Cardon et al., 1999). Accordingly, *SPL1* and *SPL12* can be considered as possible paralogues. *SPL9* and *SPL15* also appear to be possible paralogues considering their amino acid sequence similarity. The percentage of sequence identity between the members of each pair is 69 % and 64% respectively (Cardon et al., 1999).

Phenotypic examination of the mutants revealed no obvious differences with respect to wild type for *spl1*, *spl2*, *spl11* and *spl12* under normal growing conditions. However, bearing the close relation of *SPL1* and *SPL12* in mind, a redundant function of both genes seemed not unlikely. Therefore the double mutant *spl1 spl12* was created.

Preliminary data suggested an increase in size, a somewhat earlier flowering phenotype and reduced fertility in long day conditions (data not shown).
A more clear mutant phenotype however, could be observed for the two mutants *spl9* and *spl15*.

3.3 Phenotypic and Functional Analysis of the spl15 and spl9 Loss-of-Function Mutants and the Double Mutant spl9 spl15

3.3.1 Phenotypic and Functional Analysis of the spl15 Loss-of-Function Mutant

As described in the previous part, two T-DNA insertion alleles for *SPL15* were found within the SALK collection (i.e. SALK 138712 and SALK 074426). When using primers spanning the insertion site no transcript could be detected by means of reverse transcription, as shown in figure 3.6. Both T-DNA insertions led to a transcriptional block and thus most likely to no or a truncated, non-functional SPL15 protein.
Phenotypic analysis of both *spl15* alleles showed an obvious late bolting phenotype under short day growing conditions. Time to anthesis (opening of the first flower) was delayed in both of the two *spl15* alleles when compared to wild type. Interestingly, this phenotype was restricted to short day conditions. In long day, *spl15* mutants exhibited neither a delay in bolting nor in the time to anthesis (Figure 3.8.).

Spl15 mutant plants in both, late and short day conditions showed an increase in the number of rosette leaves formed by the primary shoot apical meristem (SAM) (Figure 3.8.). In long day growing conditions *spl15-1* and *spl15-2* plants on average initiated 15.9 ±1.2 and 17.2 ±2.0 rosette leaves respectively, whereas Col-0 plants appeared to develop only 13.5 ±1.7 rosette leaves on average. The mutant plants as well as the wild type on the other hand showed more or less the same number of cauline leaves i.e. 4 ±0.6 for Col-0, 4 ±0.7 for *spl15-1* and 4.5 ±0.5 for *spl15-2*.

Under short days the standard wild type initiated 47.9 ±3.5 rosette leaves. *Spl15-1* and *spl15-2* at the same time developed on average 66.5 ±3.2 and 64.9 ±4.1 rosette leaves respectively. Again the number of cauline leaves was more or less the same as for wild type (10.5 ±1.7), *spl15-1* (11 ±1) and *spl15-2* (11 ±1.2).

This increase in rosette leave number was partially due to an increased rate of rosette leaf initiation as was shown by a time course study of developing *spl15* plants (data not shown). The observation of a shortened plastochron, which describes the interval between the formation of new primordia, was also supported by the fact that *SPL15* loss-of-function plants grown in long day showed first abaxial trichomes on later rosette leaves than the typical wild-type *Arabidopsis* plant (Col-0 on leaf number 8.8 ±0.4, *spl15-2* on leaf number 10.5 ±0.5). Since the initiation of the first abaxial trichomes is fixed in terms of time, *spl15* plants produced more juvenile rosette leaves than wild type plants in the same time.

Figure 3.7.: Missing prophylls in *spl15*.
Arrowheads in the left picture indicate the propylls at the basis of two coinflorescences in the Col-0 wild type. The *spl15*-1 mutant on the right picture appears to have no prophylls at this position.

A further interesting phenotypic difference of *spl15* plants compared to wild type was the "naked" appearance of the secondary inflorescences. A closer look revealed that this impression was the result of a lack of prophylls at the basis of the coinflorescences formed in the axils of the cauline leaves (Figure 3.7.).

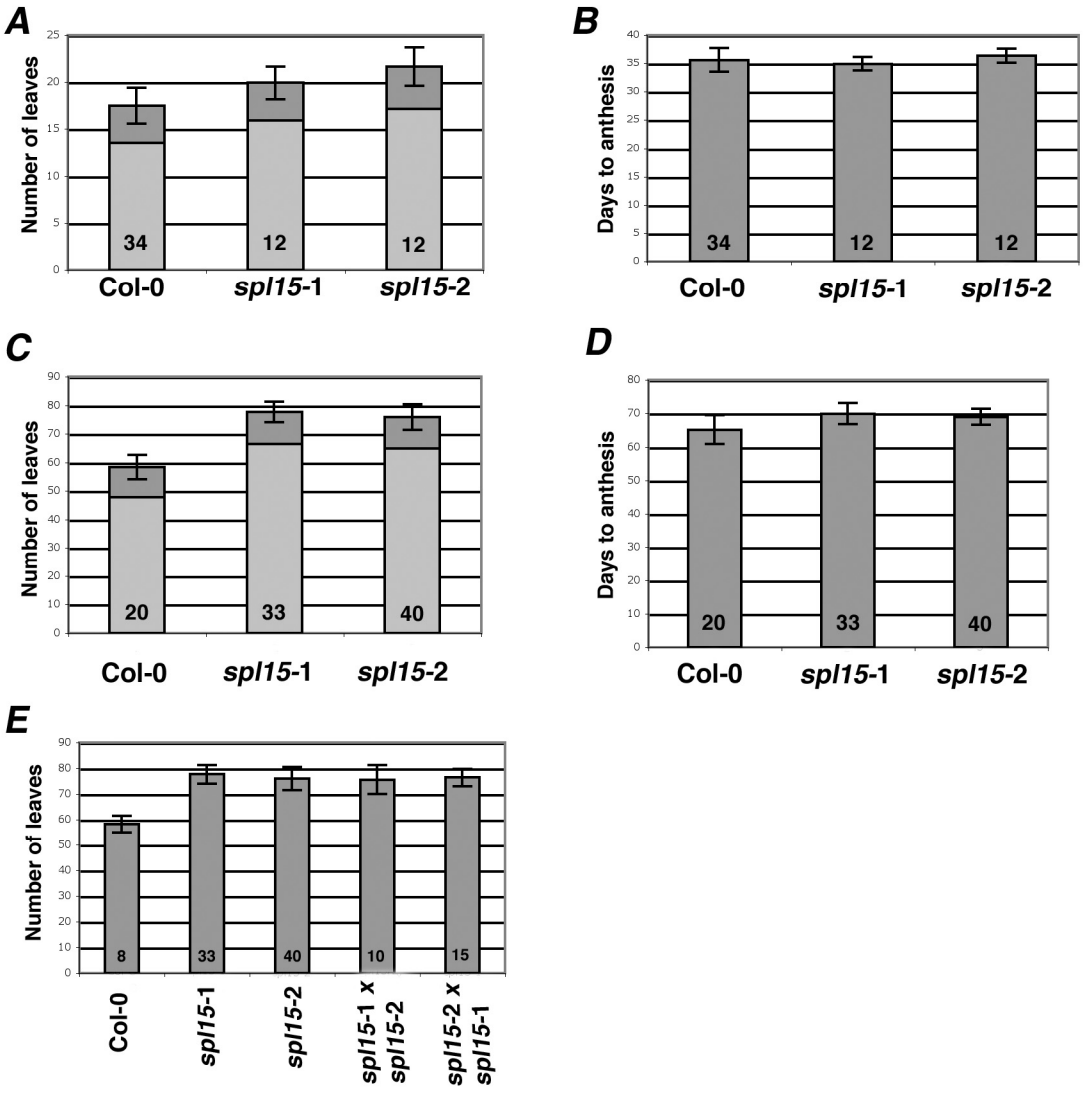

Figure 3.8.: Comparison of the number of rosette and cauline leaves and the time to anthesis between Col-0 and *spl15*.

- **(A)** Number of rosette (light grey) and cauline (dark grey) leaves in long day growing conditions. The number of leaves in both *spl15* alleles is significantly different from wild type (T test; $p<0.001$).
- **(B)** Days to anthesis in long day. T-test revealed no significant difference between the *spl15* alleles and wild type ($p>0.1$)
- **(C)** Number of rosette (light grey) and cauline (dark grey) leaves in short day growing conditions. The number of leaves in both alleles is significantly different from wild type (T test; $p<0.001$).

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(D) Days to anthesis in short day. T-test revealed a significant difference between both *spl15* alleles and wild type ($p<0.001$).
(E) Allelic test on number of total leaves in short day growing conditions: Col-0: 58.2 ±3.2, *spl15-1*: 77.7 ±3.6, *spl15-2*: 75.9 ±4.5, *spl15-1 x spl15-2*: 75.6 ±5.5, *spl15-2 x spl15-1*: 76.5 ±3.4. The T-test revealed significant differences between the *spl15* lines and wild type ($p<0.001$)

Numbers within the bars indicate the number of plants analyzed per line.
Error-bars in **(A)**, **(C)** and **(E)** indicate standard deviation of total leaf number and standard deviation of time to anthesis in **(B)** and **(D)**.

In wild-type *Arabidopsis* grown under short day, these leaves are predominant in the lower coinflorescences. However in *spl15* plants they were completely lacking.
SPL15 also seemed to affect the number of lateral roots compared to wild type. Loss of *SPL15* function led to a slight but statistically significant increase in the number of lateral roots compared to wild type (Figure 3.9.).

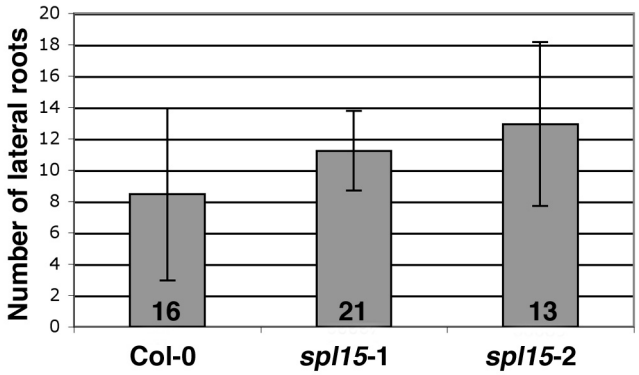

Figure 3.9.: Number of lateral roots of Col-0 in comparison to *spl15*-1 and *spl15*-2.
13 days after germination Col-0 developed 8.4±5,5 lateral roots. *Spl15*-1 and *spl15*-2 developed 11.2±2.5 and 12.9±5.2 lateral roots respectively. Students T-test revealed a significant difference between Col-0 and both mutant alleles (p-value <0.005). Between *spl15*-1 and *spl15*-2 the T-test revealed no significant difference ($p>0.1$). The numbers within the bars indicate the number of plants analyzed per line. Error-bars indicate standard deviation.

The phenotypic differences compared to wild type were observed in both alleles, *spl15*-1 as well as *spl15*-2. Nonetheless, to support that these observed differences were indeed due to a lack of SPL15 function, an allelic test was performed by crossing homozygous *spl15*-1 mutants with homozygous *spl15*-2 plants. The F1 generation of this cross, which

was heterozygous for both alleles showed the same phenotypic alterations as both homozygous parental lines (Number of total leaves in SD are shown in Figure 3.8.).

With the help of northern blot analysis, U. Unte already showed that *SPL15* can be detected in all parts of the plant except older rosette leaves (U. Unte, PhD thesis, 2003).
To get a more precise overview of the temporal and spatial *SPL15* expression pattern, a *SPL15 promoter::GUS:SPL15* (ß-glucuronidase) reporter gene construct was transformed into Col-0 and its expression was analyzed in different tissues and at different time points. Making the construct, it was taken care that the naturally occurring recognition sites for the miRNAs156 and 157 in the last exon of *SPL15* remained intact and on place.

The first sign of *pSPL15* directed GUS activity could be detected in the chalazal area of the fertilized ovule at a time when the embryo was in the globular stage (for staging see Bowman, J.L., Arabidopis: An Atlas of Morphology and Development). The embryo itself showed no GUS staining until the bend stage. Here the expression appeared to be restricted to the shoot as well as the root apical meristem and weak GUS staining could be observed within the hypocotyls (Figure 3.10.).
Later in development GUS staining became strong in the cotyledons but disappeared again when the first true leaves were formed. Throughout the seedling stage *SPL15* expression was very strong in the shoot apex and in very young leaves. GUS staining largely disappeared when leaves became older but remained detectable on the basal part of the leaf midvein. When plants started to flower weak GUS staining could be detected in the petioles and strong staining appeared in the style of the gynoceum before pollination.
In the root, the first sign of GUS staining was detected in the apical meristem of the main root. Later in development, GUS signal appeared at sites where lateral root primordia were initiated. After outgrowth, the GUS signal was strong in the meristem at the lateral roots tip as well (Figure 3.10.). In addition, a GUS signal was observed in lateral bundles as seen in Figure 3.10..

To dispel doubts about how representative the staining pattern was, the analysis was performed using two independent, homozygous lines. Both lines showed exactly the same GUS signal pattern.

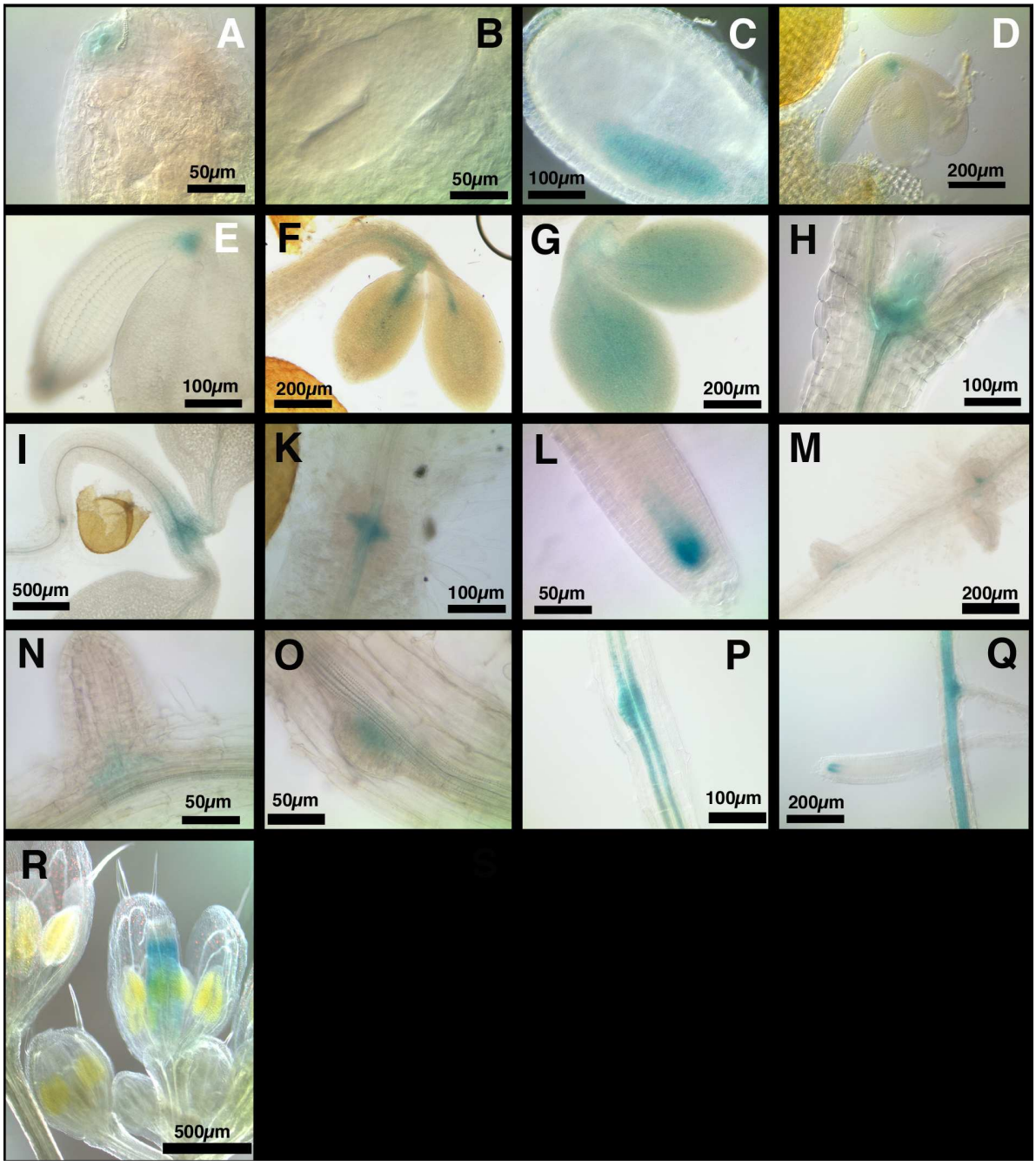

Figure 3.10.:
Analysis of *SPL15-promoter::SPL15:GUS* expression in different tissues and at different time points in *Arabidopsis* development. The first sign of GUS signal was detected at the chalazal of the fertilized ovule **(A)**. The embryo in its heart stage did not show any GUS expression **(B)**. The first signal of GUS was detected at the bend stage in the radicle of the embryo **(C)**. Later in embryo development the signal concentrated at the shoot and root apical meristems **(D, E)**. Strong GUS signal was also observed in the young cotyledons **(F, G)**. In the seedling stage the GUS expression was restricted to very young leaves **(H)**, the shoot and root apical meristem **(I, L,)** and lateral root primordia **(K, M,N, O, P, Q)**. In the flower GUS signal was observed in the style of the gynoceum **(R)** before pollination.

Since the mutant phenotype of *SPL15* and its expression pattern were to some extent reminiscent of those genes involved in auxin signaling, the staining pattern of seedlings of transgenic *pSPL15::GUS:SPL15* plants was analyzed upon induction by exogenously applied auxin. In order to do so *pSPL15::GUS:SPL15* plants were germinated and grown on medium containing the auxin transport inhibitor naphtylphthalamic acid (NPA) to block the action of endogenous auxin. After ten days the seedlings were put on medium containing 10µM of the bioactive auxin indole-3-acetic acid (IAA). The GUS staining was observed before auxin induction and four, eight, twelve and 24 hours after induction. After four hours of auxin induction the GUS signal strongly increased as compared to the signal without auxin induction. Interestingly, after eight hours the signal became weaker, even compared to non-induced plants. The reduction in signal strength was also seen after 12 and 24 hours respectively (Figure 3.11.). For the experiment two independent transgenic reporter lines were used and from each line several plants were examined.

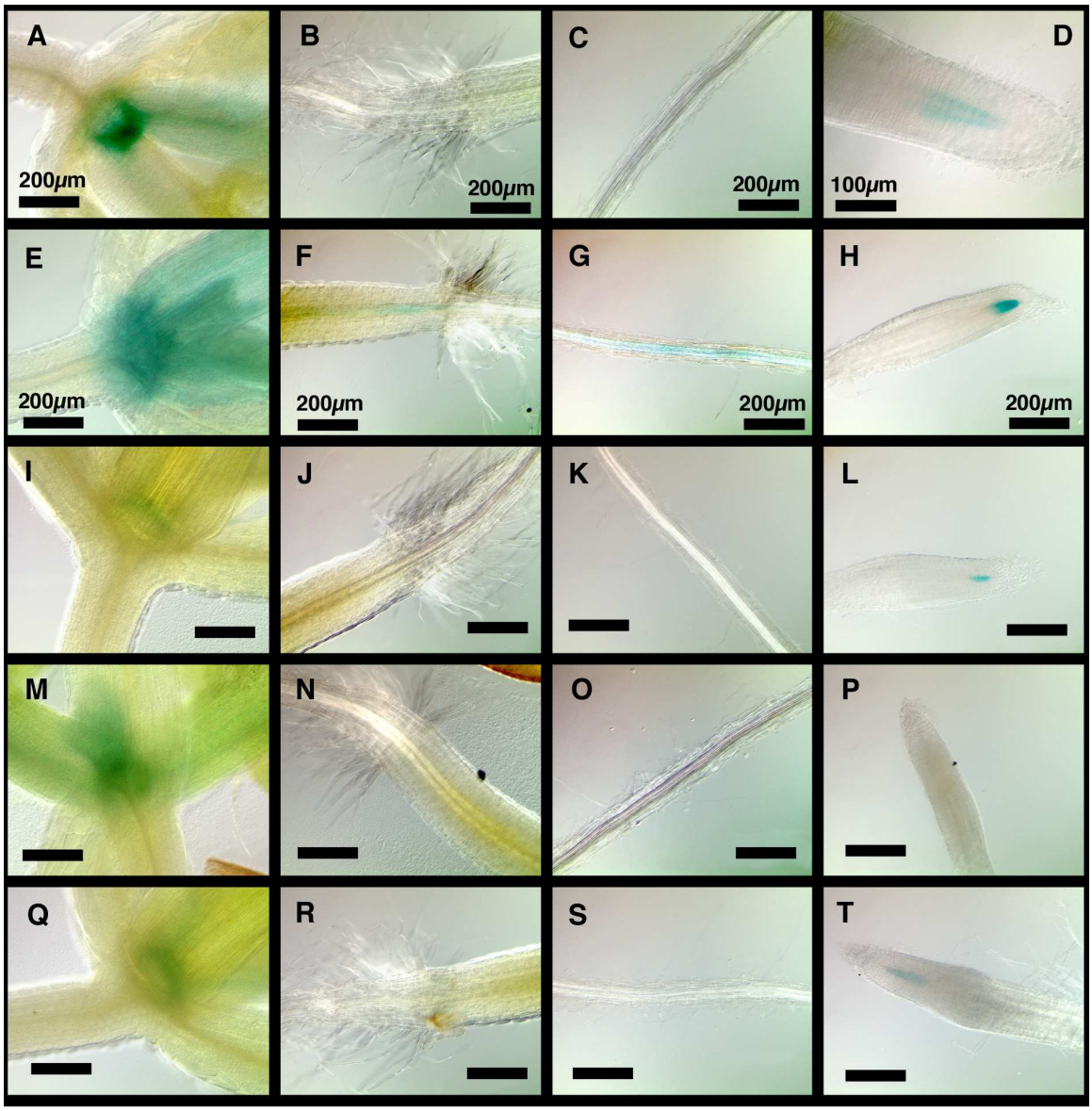

Figure 3.11.: *p::SPL15:GUS* after Auxin induction
(A-D) SAM and different parts of the root before Auxin induction
(E-H) SAM and different parts of the root after four hours of Auxin induction
(I-L) SAM and different parts of the root after eight hours of Auxin induction
(M-P) SAM and different parts of the root after 12 hours of Auxin induction
(Q-T) SAM and different parts of the root after 24 hours of Auxin induction

The SBP-box gene family in Arabidopsis consists of 17 members, eleven of which are targeted by the miRNA156 and the very closely related miRNA157. Among the targets of the miRNA156 is *SPL15*. The GUS transcript generated from the *pSPL15::GUS:SPL15* transgene is supposed to have the miRNA156 target site included (confirmed by RT-PCR, result not shown). Hence the GUS staining mirrors not just *SPL15* transcriptional activity as regulated by cis-acting elements in the promoter but also on the translational level as regulated by the miRNA156/157.

To find out more about the temporal and spatial expression of the miRNA156/157, a *SPL15-YFP* reporter construct under the control of the viral, constitutive 35S promoter was transformed into Col-0 plants.

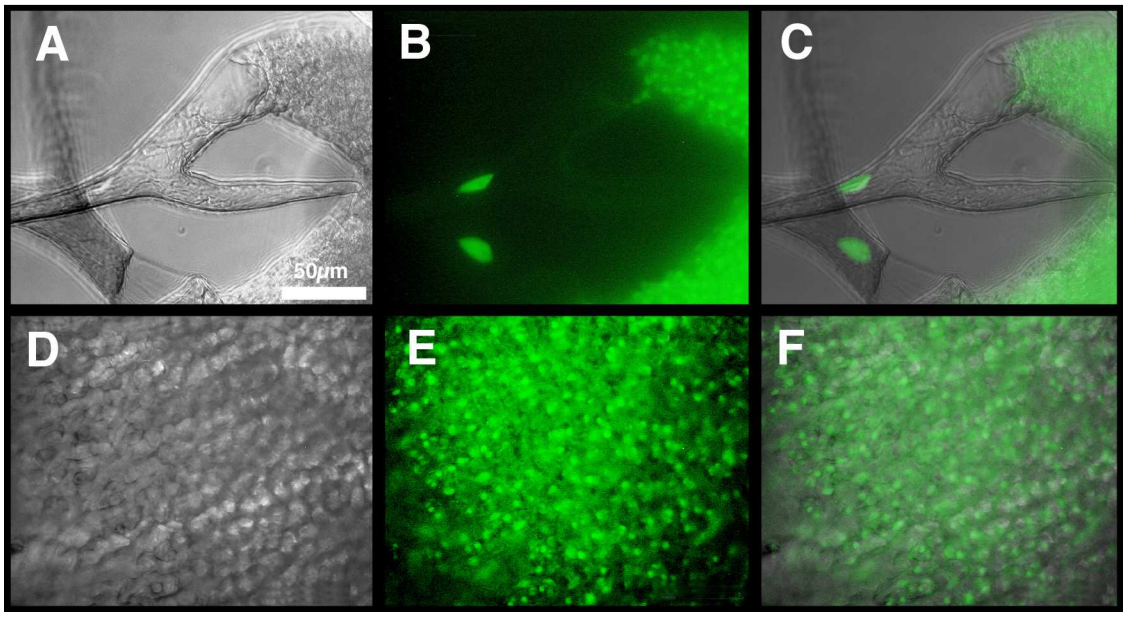

Figure 3.12.:
35S::SPL15:YFP in trichomes **(A, B, C)** and leaf cells **(D, E, F)**. In both, in the trichomes as well as in the leaf cells the signal is very much localized to the nucleus.

Figure 3.12 shows *SPL15-YFP* expression in trichomes as well as in leaf cells. The expression was localized in the nucleus which is expected for a putative transcription factor. Interestingly, the strength of the YFP signal positively correlated with the severeness of particular phenotypic alterations (Figure 3.13.).

Plants that showed elevated levels of YFP signal were smaller, had serrated, narrow rosette and cauline leaves and were characterized by an overall slower development. Also the number of rosette leaves was greatly reduced.

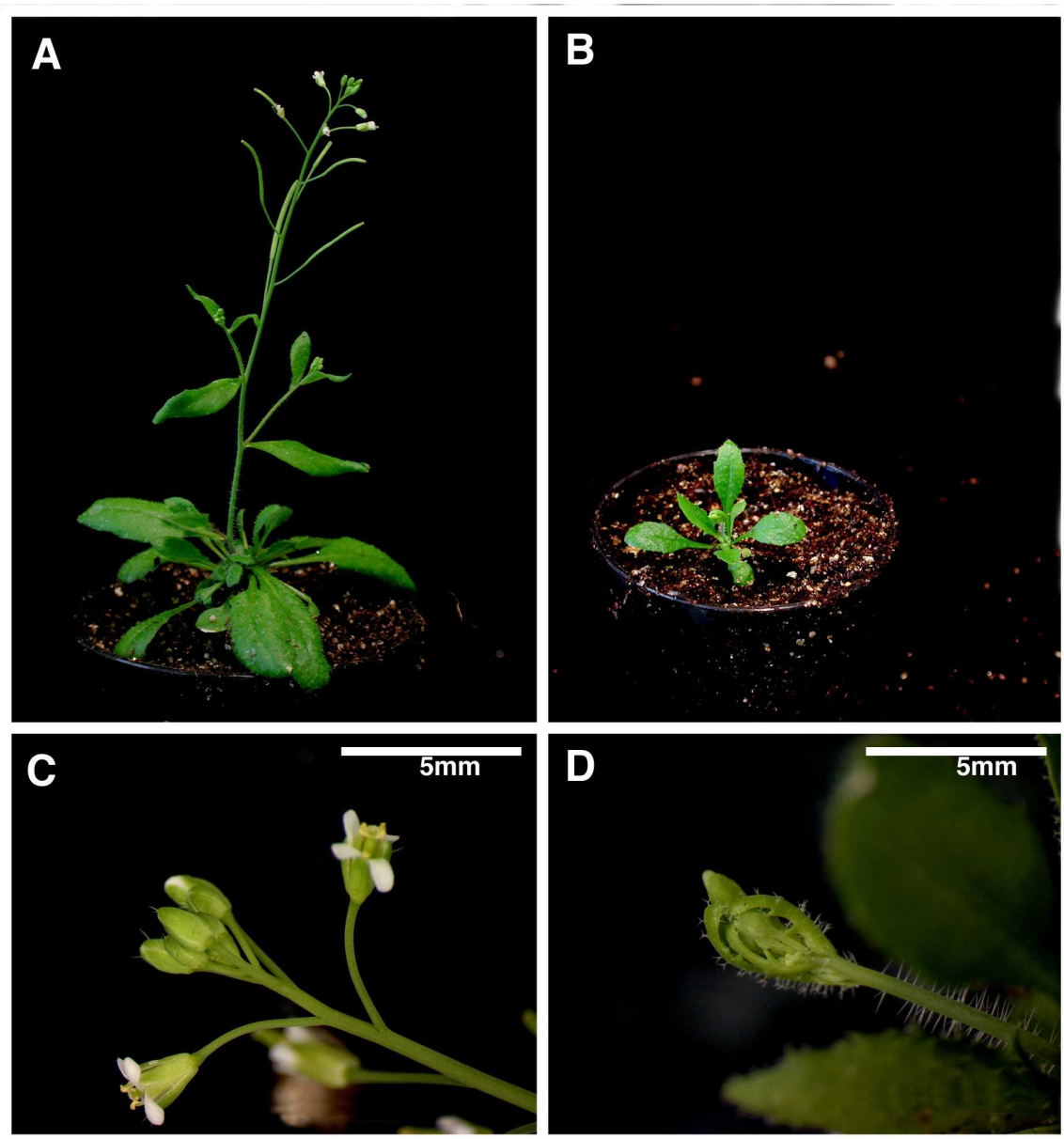

Figure 3.13.:
Phenotypic analysis of F1 plants expressing a *35S::SPL15:YFP* reporter construct. Among the F1 population, plants with wild type like phenotype **(A)** and **(C)** as well as plants with severe phenotypical alterations **(B)** and **(D)** were observed. Interestingly, plants showing phenotypic alterations (i.e. more narrow and serrated rosette and cauline leaves, abnormal flowers) also have a stronger YFP signal suggesting stronger expression of the transgene.

3.3.2 Phenotypic and Functional Analysis of the *spl9* Loss-of-Function-Mutant

SPL9 is the closest relative of *SPL15* within the *SPL* gene family with respect to its sequence and according to phyllogenetic analysis.

Loss of *SPL9* function leads to a loss of apical dominance (U. Unte, PhD thesis, 2003). Furthermore *spl9*-1 plants showed, as previously described for *spl15* mutants, an increased number of rosette leaves in long as well as in short day conditions (Figure 3.14.). Col-0 on average developed 13.5 ±1.7 rosette leaves in long days whereas *spl9-1* initiated 16.3 ±1.8 rosette leaves. In short day conditions Col-0 made 47.9 ±3.5 rosette leaves. In contrast, 52.6 ±3.3 rosette leaves were counted for the *spl9*-1 mutant when grown under similar conditions. The number of cauline leaves in long days was about 4 for both Col-0 (±0.6) as well as for *spl9*-1 (±0.7). In short day Col-0 developed 10.5 ±1.7 cauline leaves and the *spl9*-1 mutant 12 ±1.8.

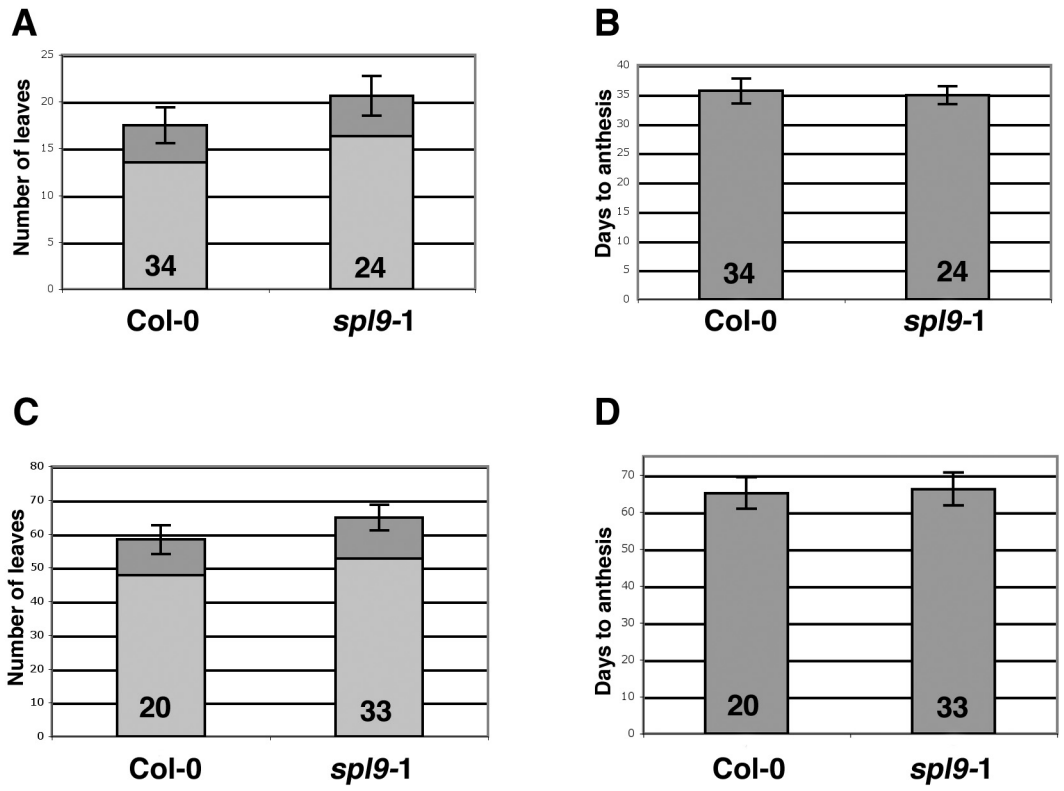

Figure 3.14.: Comparison of the number of rosette and cauline leaves and the time to anthesis between Col-0 and *spl9-1*.

- **(A)** Number of rosette (light grey) and cauline (dark grey) leaves in long day growing conditions. The number of leaves in the *spl9* plants significantly differed from wild type (T-test; $p<0.001$).
- **(B)** Days to anthesis in long day. No significant difference was revealed. T-test; $p>0.05$
- **(C)** Number of rosette (light grey) and cauline (dark grey) leaves in short day growing conditions. The number of leaves in the *spl9* plants significantly differed from wild type (T-test; $p<0.001$).
- **(D)** Days to anthesis in short day. No significant difference was revealed. T-test; $p>0.05$

Error-bars in (**A**) and (**C**) indicate standard deviations of total leaf number. Error-bars in (**B**) and (**D**) also indicate standard deviations. The numbers within the bars indicate the number of plants analyzed.

Again, time to anthesis appeared to be completely unaffected. On average Col-0 plants and *spl9* mutants opened their first flower at the same day 36 ±2.1 days for Col-0 and 35 ±1.5 days for spl9-1 in long day); and 65 ±4.3 days for Col-0 and 66 ±4.5 days for spl9-1 on short day). Preliminary results of a second loss-of-function allele (*spl9*-2) showed the same phenotype than what was described for *spl9*-1 (data not shown).

SPL9, like *SPL15,* has a recognition site for the miRNAs156 and 157 in its last exon.

A *SPL9-YFP* construct under the control of the strong constitutive 35S promoter was transformed into *Arabidopsis* Col-0 plants. Among the T1 transformants some plants showed phenotypic differences on comparison to wild-type plants. Again as observed for *SPL15:YFP*, a strong correlation between the severeness of phenotypic alterations and YFP signal strength could be observed (Figure 3.16.). Plants that showed a strong YFP signal showed strong phenotypical alterations, whereas plants showing only a weak or no signal were phenotypically indistinguishable from wild-type plants.

As determined by means of RT-PCR, the strong YFP signal on the one hand and the phenotypical alterations on the other also strictly correlated with the amount of transcript that could be amplified (Figure 3.16.).

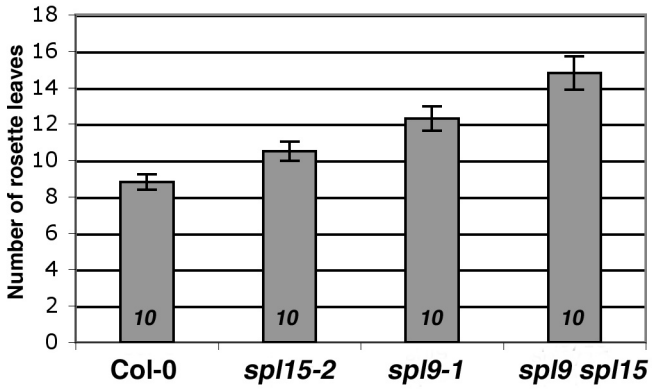

Figure 3.15.: Rosette leaf with first abaxial trichome.
Col-0: 8.8 ±0.4; *spl15*-2: 10.5 ±0.5; *spl9*-1: 12.3 ±0.7; *spl9 spl15*: 14.8 ±0.9
The T-test revealed significant differences of all mutant lines compared to wild type ($p<0.001$).
The numbers within the bars indicate the number of plants analyzed. Error-bars indicate standard deviation.

The phenotypic differences of the *SPL9-YFP* overexpressor plants compared to wild-type plants were similar to what had been observed with the *SPL15-YFP* construct. Again the plants were far behind the wild type with respect to development. Also, the rosette as well as the cauline leaves appeared to be extremely narrow and serrated. The overall impression of the phenotype was that the alterations were similar but more severe to what had been observed with the *SPL15-YFP* plants.

In particular the flower was severely altered.

In order to complement the loss of SPL9 in the mutant, a genomic fragment spanning the entire *SPL9* locus was transformed into the *spl9* mutant. Within the T1 generation plants with wild-type like phenotype were found but some plants showed clear phenotypical alterations that again were very similar to what had been observed in plants overexpressing *SPL9-YFP* or *SPL15-YFP*. The severity of the alterations positively correlated with the expression level of the transgene. As shown in figure 3.17. plants that appeared to have more transgene expression also showed phenotypes different to wild type. Rosette leaves and cauline leaves were narrow and serrated.

Because of their close relationship SPL9 and SPL15 were expected to display functional redundancy. Therefore a *spl9 spl15* double knock-out mutant was created.

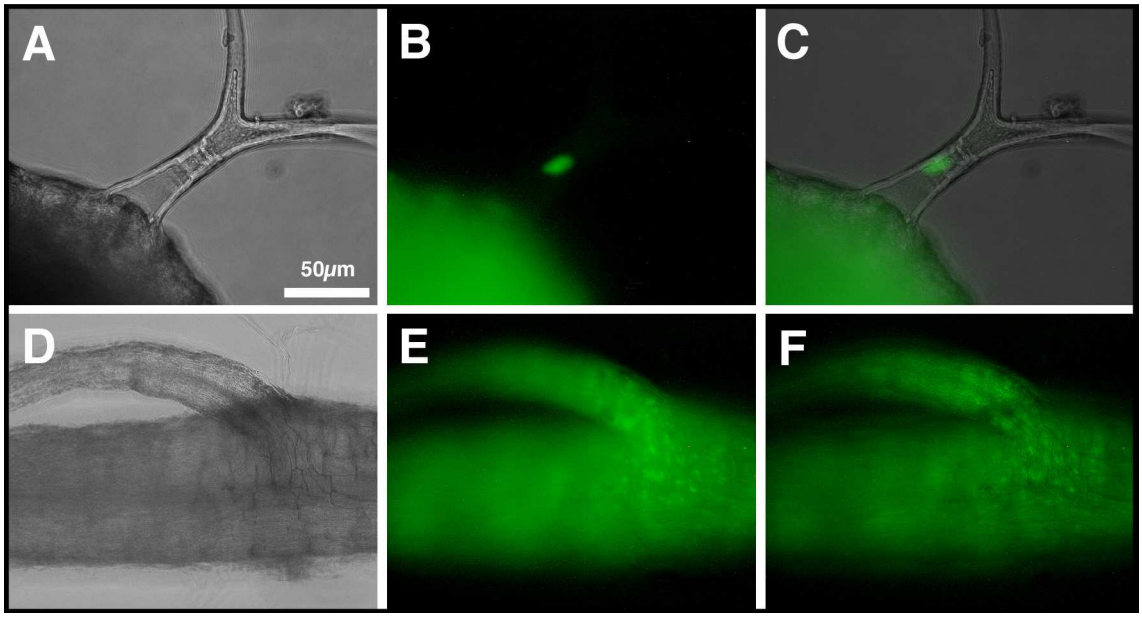

Figure 3.16.:
Analysis of the YFP signal of transgenic plants expressing ***SPL9:YFP*** under the control of the strong 35S promoter. The signal is nucleus specific as is shown in **(A), (B), (C)** for trichomes and for root cells in **(D)**, **(E)** and **(F)**.

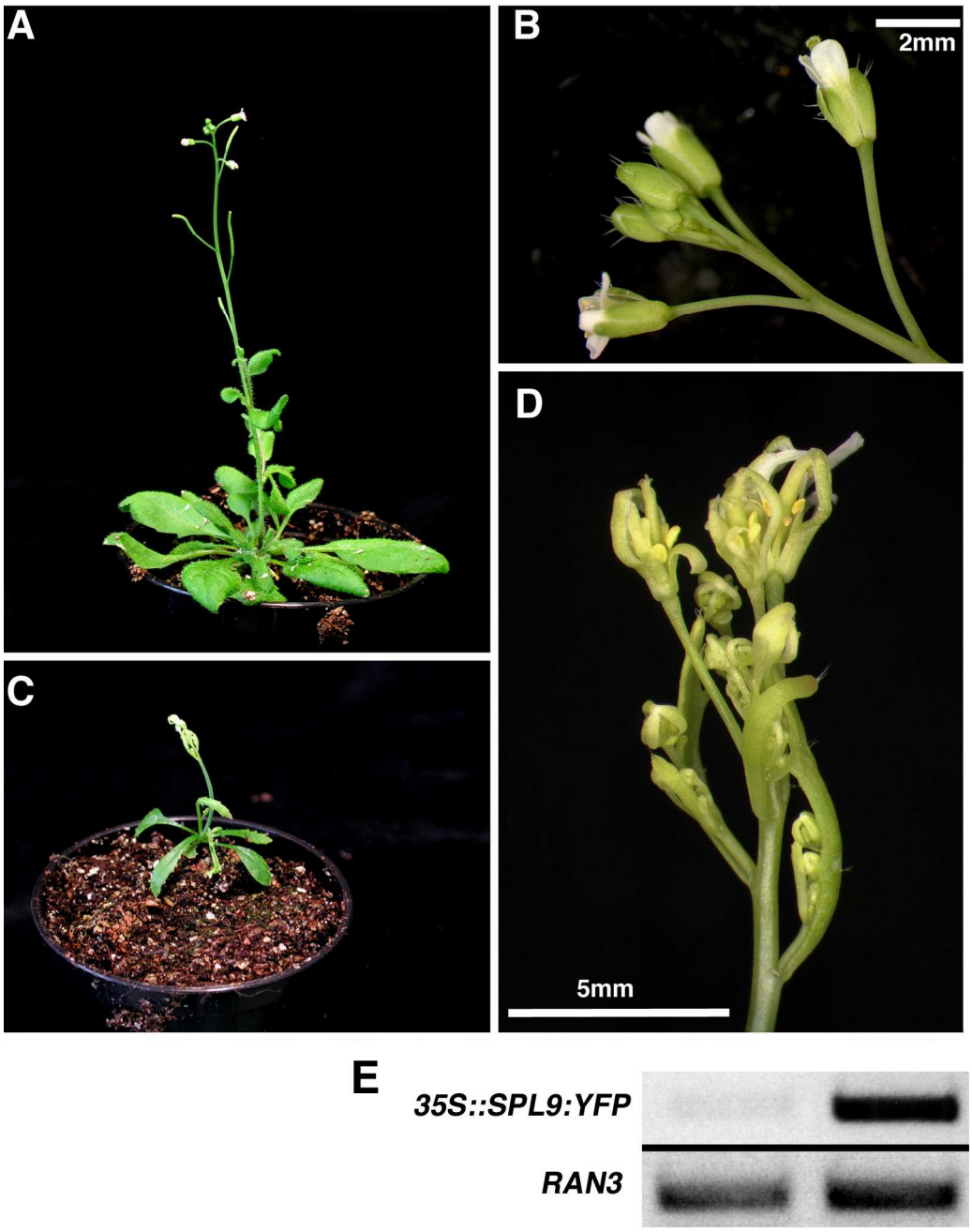

Figure 3.17.:
Phenotypic analysis of the F1 population of transgenic plants expressing a *SPL9:YFP* reporter construct under the control of the strong constitutive viral 35S promoter. Note the phenotypic differences among the F1 plants. **(A)** and **(B)** shows a plant which appears to be more or less wild-type like whereas **(C)** and **(D)** shows one example of a plant with severe phenotypical abnormalities. Plants are dwarfed, develop less but more narrow rosette leaves and are sterile. The phenotype strictly correlates positive with the amount of transgene expressed. In wild-type like plants almost no transgene expression can be detected by RT-PCR ((**E**), left band) whereas in plants with severe phenotypical defects, transgene expression is high ((**E**), right band).

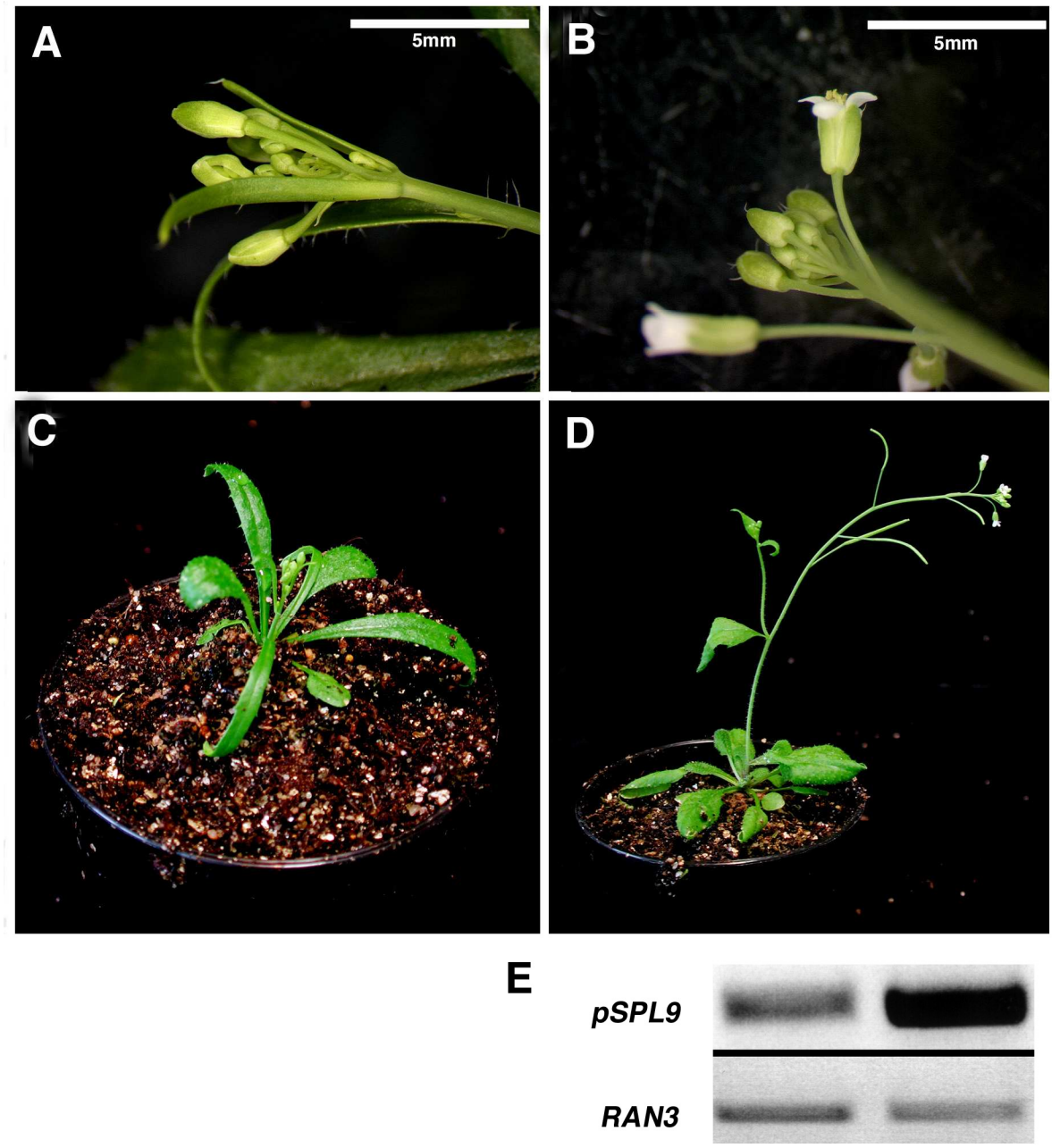

Figure 3.18.: Phenotypic analysis of the F1 generation of transgenic *spl9-1* plants expressing *SPL9* under its natural promoter in order to complement the *spl9* phenotype. Note the phenotypic differences among the F1 population. **(C)** and **(D)** shows a plant with a wild-type like phenotype whereas **(A)** and **(B)** shows a plant with clear phenotypical alterations, i.e. a reduced number of rosette leaves, very narrow rosette and cauline leaves and a overall slower development. Interestingly, plants showing the described altered phenotype appear to have more *SPL9* transcript ((**E**), right band) than wild-type looking plants ((**E**), left band).

3.3.3 Phenotypic and Functional Analysis of the *spl9 spl15* Loss-of-Function Double Mutant

Figure 3.19.: Phenotypic comparison of Col-0, *spl9 spl15* and *35S:miRNA156b*.
All plants were grown in long day conditions. Note the increase of the number of rosette leaves and the loss of apical dominance of the *spl9 spl15* double mutant which is clearly distinct from the wild type and similar but not as severe as of the miRNA156b overexpressing plant.

Loss of *SPL9* as well as of *SPL15* function led to severe phenotypical alterations compared to wild type plants. *Spl9* plants were characterized by a bushy and stocky appearance. *Spl15* instead was later bolting than wild type and lacked prophylls at the base of the coinflorescences. Both knock outs have in common that they initiated rosette leaves faster than wild type. Very intriguingly the double knock out *spl9 spl15* appeared to show an additive effect of the single mutant phenotypes. The loss of apical dominance that was most apparent in the *spl9* mutant appeared to be even more severe in the *spl9 spl15* double mutant. The initiation rate of rosette leaves also increased in the double knock out. On average s*pl9 spl15* plants showed 21.6 ±2.3 rosette leaves in long days compared to an average of 16.3 ±1.8 for the single *spl9* mutant and an average of 15.9

±1.2 for the single *spl15* mutant. Col-0 developed 13.5 ±1.7 rosette leaves on average. In short day conditions *spl9 spl15* plants developed about 76 ±4.2 rosette leaves whereas *spl9* and *spl15* single mutants developed only 52.6 ±3.3 and 66.5 ±3.2 rosette leaves on average. Col-0 initiated an average of 47.9 ±3.5 rosette leaves in short day. The number of cauline leaves counted was more or less similar for all plants in any condition (Figure 3.20.). Time to anthesis of the double knock-out *spl9 spl15* did not significantly differ to any of the single mutants or to wild type.

The first rosette leaf in the *spl9 spl15* mutant where abaxial trichomes could be detected was the leaf number 15 ± 0.9, about six more leaves than in Col-0 (8.8 ±0.4) (Figure 3.15)

As mentioned before 11 out of the 17 *SPL* genes in *Arabidopsis* are targeted by the microRNA156. Schwab et al. overexpressed the MIRNA156b in Col-0 background and could show by means of micro array technology that 10 *SPL* genes targeted by the miRNA156 and represented on the ATH1 array are significantly down regulated in the early inflorescence apex (Schwab et al., 2005).

Consistent to the phenotypes observed for the *spl9 spl15* double knock outs overexpressing miRNA156b causes a faster initiation of rosette leaves and a severe decrease in apical dominance (Schwab et al., 2005).

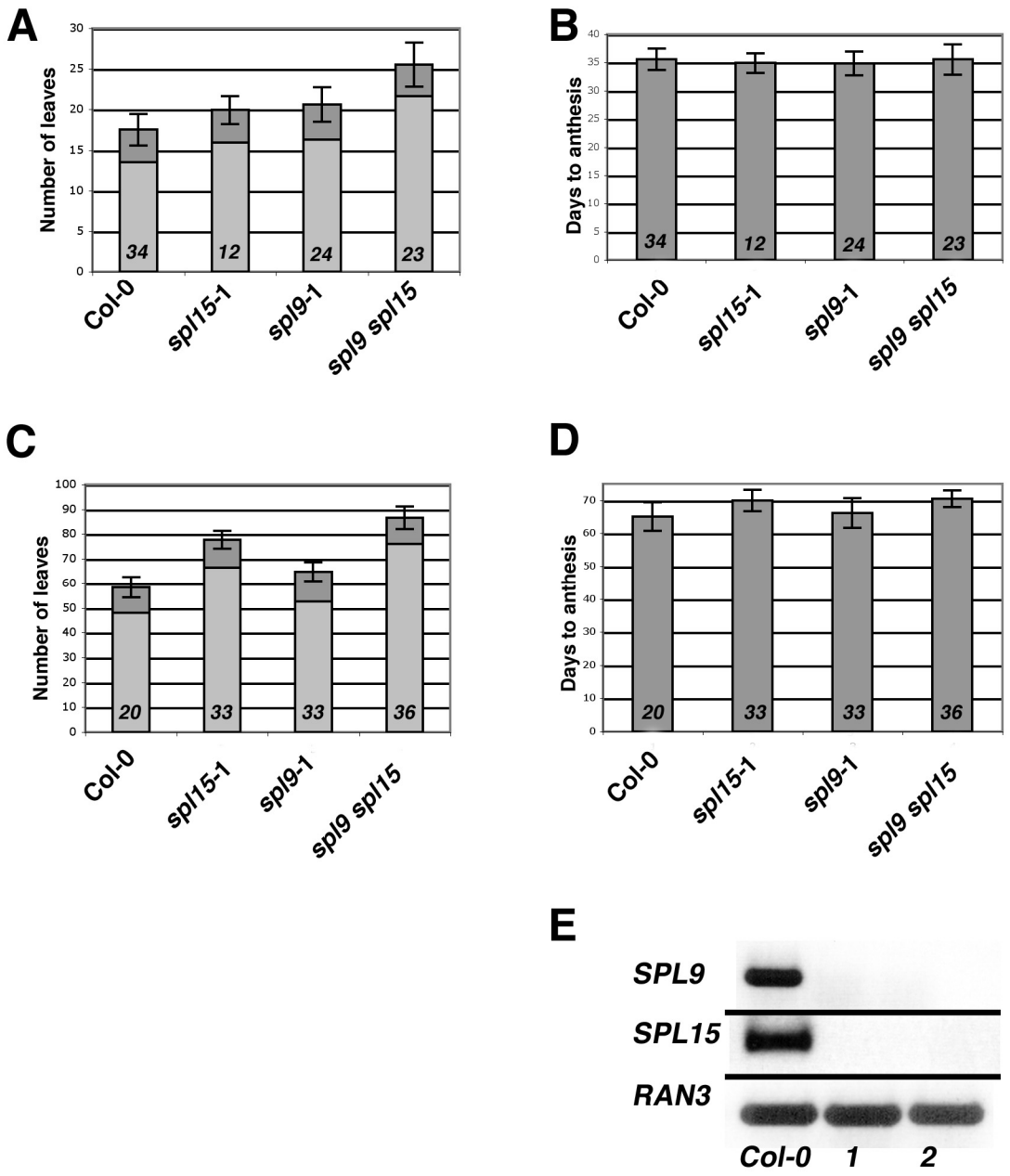

Figure 3.20.: Comparison of the number of rosette and cauline leaves and the time to anthesis between Col-0, *spl15-1*, *spl9-1* and *spl9 spl15*.

- **(A)** Number of rosette (light grey) and cauline (dark grey) leaves in long day growing conditions. The t test revealed significant differences of all mutant line compared to wild type ($p<0.001$).
- **(B)** Days to anthesis in long day. Col-0: 35.6 ±2.1, *spl15*-1: 34.9 ±1.2, *spl9*-1: 34.8 ±1.5, *spl9 spl15*: 35.5 ±1.6. The T-test revealed no significant differences of the mutant lines compared to wild type ($p>0.05$).
- **(C)** Number of rosette (light grey) and cauline (dark grey) leaves in short day growing conditions. The T-test revealed significant differences of all mutant lines compared to wild type ($p<0.001$).

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(D) Days to anthesis in short day.Col-0:65.1 ±4.3; *spl15*-1: 69.9 ±3.2; *spl9*-1: 66.2 ±4.5; *spl9 spl15*: 70.5 ±2.5. The t test revealed no significant difference of *spl9* compare to wild type ($p>0.5$) but significant differences of the *spl15* mutant and the *spl9 spl15* double mutant compared to wild type ($p<0.001$).

(E) RT-PCR revealed neither for *SPL9* nor for *SPL15* transcript in the two *spl9 spl15* double knock out lines (1 and 2) as compared to wild type.

The numbers within the bars indicate the number of plants analyzed. Error-bars indicate standard deviation.

4 Discussion

4.1 Identification of Downstream Target Genes of the Transcription Factor SPL8

The transcription factor SPL8 is a member of the SBP-box gene family in *Arabidopsis* and is required for the proper initiation of microsporangium formation within the developing anthers and for the regular entrance of spore mother cells into meiosis (Unte et al., 2003). Furthermore, a reduced number of trichomes on the sepals in *spl8* mutant plants and an increased number of trichomes in *SPL8* overexpressing plants (Unte et al., 2003, Zhang, 2005) suggest an important role in trichome formation on sepals.
Based on phyllogenetic analysis *SPL8* can be grouped together with the other middle-sized *SPL* genes, although it is the only member of this class that has no miRNA156/157 recognition site.

Consistent with the above described mutant phenotypes, previous RNA gel blot analysis performed on poly(A)$^+$ RNA from aerial tissues of long day grown plants revealed highest *SPL8* expression in young inflorescences formed after the floral transition (Cardon et al., 1997). *In situ* hybridization on young flower buds revealed high *SPL8* expression in developing pollen sacs, up to anther stage 6. Low expression was detected in other floral tissues (Unte et al., 2003). These findings positively correlate with the aberrant *spl8* mutant anthers and the observation that the mutants develop fewer trichomes on the sepals.

4.1.1 Global expression profiling revealed putative *SPL8* target genes

In this study, genes that are assumed to be controlled by the SBP-box transcription factor SPL8 and to be directly or indirectly involved in the above described processes of flower development were identified by global expression analysis with the help of the micro-array technology.

A N-terminal fusion of SPL8 to the viral activation domain VP16 under the control of an alcohol inducible promoter was used in order to temporally overexpress *SPL8* in two weeks old, long day grown plants by an ethanol inductive pulse. The comparison of gene expression in *VP16:SPL8* overexpressing plants and in control plants revealed 28 putative *SPL8* target genes that became highly expressed upon activation of the *VP16:SPL8* transgene.

The cut-off level was set at $2^{1.5}$ fold. The highest increase in expression was measured for the GDSL-motive lipase/hydrolase family protein At5g45960 with $2^{39.5}$ fold (Table 3.1.).

Verification of the micro-array results by means of semi-quantitative RT-PCR revealed three false positive genes. At3g57520 and At3g50770 were activated by the alcohol receptor AlcR rather than through the activity of the *VP16:SPL8* transgene because their expression level increased upon ethanol induction also in plants that were only transgenic for the alcohol receptor gene. The gene At1g69760 which shows a differential expression of four fold (2^2) in the chip experiment did not show any differential expression in the RT-PCR.

Unfortunately, because of technical reasons, five genes (i.e. At1g56150, At3g53950, At1g22030, At4g19380 and At1g69760) could not be amplified by RT-PCR and therefore the differential expression revealed by the micro-array could not be verified.

The remaining 23 genes showed the expected differential expression although the values measured by RT-PCR on the one hand and in the micro-array experiment on the other hand could not be compared quantitatively because of limited accuracy of the semi-quantitative RT-PCR. In the future quantitative real-time PCR should solve this problem.

4.1.2 GTAC binding motif is overrepresented in the promoter region of target genes

Previous random primer selection experiments revealed that the SBP-domain of SPL8 binds to the GTAC core sequence (Birkenbihl et al., 2005). Thus, in the promoter sequence of the 28 putative SPL8 target genes, the GTAC motif should be present or even overrepresented compared to a random promoter set.

Computational analysis with the help of the programme PROMOMER revealed that in fact the GTAC motif is underrepresented compared to the statistically expected value for a tetranucleotide about two fold in any random promoter set.
This remarkable result was as well discovered previously with the help of the programme PATMATCH (Birkenbihl et al., 2005).
Interestingly, in the promoter set of the 28 putative SPL8 target genes, the GTAC motif was about two fold overrepresented compared to the random set and actually occurred as often as it is statistically expected for any tetranucleotide.
The fact that the GTAC motif is not evenly distributed throughout all promoters but rather concentrated on the promoters of only a few genes clearly shows its importance and suggests an evolutionary pressure against a random distribution.
These findings thus support the conclusion that the conducted micro-array analysis indeed resulted in the identification of *SPL8* target genes.

4.1.3 Further RT-PCR based test can narrow down the number of putative target genes

The expression profiling was based on an artificial situation. Total RNA was extracted from rosette leaves of vegetative plants, a tissue where *SPL8* expression is normally absent. To account for this problem, SPL8 was fused to the viral activation domain VP16. As discussed before, one could imagine that fusion to the activation domain leads to unspecific binding and therefore to the upregulation of unspecific target genes. Furthermore, due to the activating action of the VP16 domain, genes that would normally be repressed by SPL8 can not be distinguished from genes that become activated under natural conditions.
For the subsequent RT-PCR that was done to confirm the array results the same total RNA was used that was hybridized onto the chip. The RT-PCR therefore was only independently confirming that the micro-array experiment worked technically. By no means however had the RT-PCR proofed that the selected candidate target genes indeed are activated by SPL8 under natural conditions. Therefore, in a second RT-PCR based experiment the expression of the presumed SPL8 targets were examined on total RNA

extracted from young floral buds of wild-type plants, *spl8* knock-out plants and *SPL8* overexpressors.

The correlation between *SPL8* expression and the expression of the putative target genes should be unambiguous. If SPL8 acts as an activator, the expression level of its target genes are expected to be high in the overexpressors, low in spl8 mutant plants and intermediate (i.e. normal) in the wild type. If, in contrast, SPL8 acts as a repressor the expression level of target genes are expected to be increased in the mutant, low in the *SPL8* overexpressor and probably intermediate in wild type.

Out of all tested presumed SPL8 target genes, none fulfilled the above described criteria as being repressed by SPL8. On the other hand several genes appeared to have an expression profile as it would be predicted for genes activated by SPL8 suggesting that SPL8 acts as a transcriptional activator.

The genes At5g45960 (GDSL lipase/hydrolase protein), At3g10570 (Cytochrome P450) and At3g63240 (Endo-/exo-/phosphatase family protein) were expressed predominately in young floral tissue (Figure 3.4.). In addition the expression levels positively correlated with the expression level of *SPL8* i.e. were high in the *SPL8* overexpressor, low in the *spl8* mutant and intermediate in the wild type. The data strongly suggest that *SPL8* is able to activate the above described genes. Whether this activation is direct or indirect via further transcription factors can not be determined.

Other genes, namely At5g27920 (F-box protein), At5g61460 (Structural maintenance of chromosomes family protein) and At3g15270 (*SPL5*) showed a similar expression in floral tissue but were in addition upregulated in the vegetative part of the *35S:SPL8* plants as well (Figure 3.4.). The data indicate that those genes are probably activated by SPL8 but apparently floral tissue specific co-factors are not necessary for their activation.

Gene expression of At5g14230 (ankyrin repeat family protein) also seems to be induced by *SPL8* but in this case exclusively in the vegetative plant tissue indicating that induction of this gene may be an artifact.

4.1.4 Function of some potential *SPL8* target genes suggest role in anther development

Most identified potential SPL8 target genes are not yet characterized and hence of unknown biological and molecular function (see Appendix). However for some of the identified genes a role in plant development has been described.

The GDSL lipase At5g45960 appeared to have the strongest increase in expression upon *SPL8* induction. GDSL lipases are hydrolytic enzymes with multifunctional properties. In plants, GDSL lipases may play an important role in the regulation of morphogenesis and development, particularly in the degradation of plant cell walls (Brick et al., 1995). Interestingly the expression level of At5g45960 is highest in the flower with a tendency to be higher in older flowers (Schmid et al., 2005; see Appendix C).

The micro-array experiment revealed two cytochrome P450 genes, At3g10570 and At3g26200. Cytochrome P450 enzymes have been reported to play an important role in *Arabidopsis* fertility. CYP74A for example is an important enzyme involved in the jasmonic acid biosynthetic pathway. Loss-of-function mutants in this gene showed severe male fertility defects (Park et al., 2002; von Malek et al., 2002).

At5g27920 has been reported to be related to brassinosteroids (BR); (Lisso et al., 2005) which makes it an interesting potential SPL8 target. BRs are highly potent growth-promoting plant hormones that are essential for anther development. For example *dwf4* and *dwarf1* exhibit a reduction in stamen filament elongation leading to sterility (Azpiroz et al., 1998; Choe et al., 1999).

The SBP-box gene *SPL5* has shown to be strongly expressed in flowers (Cardon et al., 1999; Schmid et al, 2005) and could thus well represent a natural *SPL8* target.

The annotation of At1g56150, At5g27920 and At1g16510 suggest a role in auxin mediated developmental processes. Interestingly, Licoln and colleagues have reported that *auxin-resistant1 (axr*1) produce less pollen and the stamen filaments fail to elongate, resulting in reduced male fertility (Lincoln et al., 1990).

At1g08040 has been annotated as being involved in ethylene biosynthesis. This is interesting because disturbed ethylene concentration and tissue sensitivity has been reported to affect anther dehiscence (Rieu et al., 2003).

In order to help placing *SPL8* in a regulatory network responsible for anther development in *Arabidopsis*, further experiments are necessary. Temporal and spatial expression pattern of the potential target genes should be determined using RT-PCR and *in situ* hybridization. Promoter deletion studies should be performed to test the functionality of the found GTAC motifs. In addition the physical interaction of SPL8 with the GTAC motifs within the target gene promoter regions should be analyzed using chromatin-imunoprecipitation (X-ChIP). Furthermore, mutant analysis of the target genes should give an insight in the pathway, *SPL8* is acting in.

4.2 Reverse Genetics revealed possible *SPL* gene functions

Reverse genetics was used as the method of choice in order to gain more insight into the biological function of the *SPL*-genes in *Arabidopsis thaliana.* Publicly available T-DNA insertion collections were screened for T-DNA insertions in the *SPL*-genes. RT-PCR revealed that insertions in *SPL1*, *SPL2*, *SPL9*, *SPL11*, *SPL12* and *SPL15* caused aborted transcription. *SPL1* and *SPL12* belong to the sub-class of large genes within the *SPL*-gene family. In contrast, *SPL2*, *SPL9*, *SPL11* and *SPL15* belong to the subfamily of mid-sized members and have a miRNA156/157 recognition site present in the coding region of their last exon.

Both the *spl2* and *spl11* mutants did not show any obvious phenotypic change in comparison to wild type. One possible explanation could be functional redundancy. *SPL10*, as a probably evolutionary very close relative to *SPL11*, e.g. both genes share 78% sequence identity and lay immediately next to another on chromosome number 1 (Cardon et al., 1999), could have redundant functions and therefore mask the knock-out phenotype. Redundant functions of *SPL10* could also explain the non-existing mutant phenotype of *spl2* since phyllogenetic analysis shows a close relation of *SPL10* to *SPL2* as well. Another possibility for the lack of a *spl2* and *spl11* loss-of-function phenotype may be that the growing conditions under which the plants were observed did not lead to

phenotypic changes. For this reasons, the two mutants were not followed up further in this study.

4.2.1 SPL1 and *SPL12* have redundant functions and may be involved in controlling copper homeostasis

SPL1 and *SPL12* are closely related according to phyllogenetic analysis and therefore possible paralogues. They both belong to the large *SPL* genes in *Arabidopsis*.

For *SPL1* as well as for *SPL12*, a T-DNA insertion line could be identified within the SALK collection. In both cases the T-DNA inserted in the coding region of the last exon. RT-PCR revealed for *spl1* as well as for *spl12* a partial transcript which stops at the 5' border of the T-DNA. Phenotypic analysis of the two mutants revealed no obvious difference to wild-type plants. It can not be excluded that the partial transcription products, despite lacking the 3' end, resulted in a truncated protein with some function retained.

Alternatively, the lack of a mutant phenotype may also be explained by the fact that the two paralogue genes display functional redundancy. Hence a double knock-out was created and analyzed phenotypically. Preliminary results point towards a weak early flowering phenotype, an increase in rosette leaf size and reduced fertility in long day conditions, indicating that these genes indeed have redundant functions.

So far, within the subfamily of large *SPL* genes there is only the *spl14* mutant phenotype described in *Arabidopsis* (Stone et al., 2005). In addition to resistance to the fungal toxin Fumonisin B1, the *spl14* mutant displays elongated petioles and enhanced leaf margin serration compared with wild type. In contrast to the somewhat earlier flowering phenotype of the *spl1 spl12* double mutant, transition to flowering has been reported to occur a few days later in the *spl14* mutant than in wild-type plants (Stone et al, 2005).

Recently it has been shown that *CRR1*, a *Chlamydomonas* specific SBP-box gene (Kropat et al., 2005) is a key regulator of copper homeostasis (Quinn et al., 1995). In situations of copper-deficiency Crr1 activates certain target genes (i.e. *CYC6*, *CPX1*, *CRD1* and *CTR*) through binding to cis-acting copper-response elements (CuRE) in their promoter regions. Mutational analysis of the promoters on the *CYC6* and *CPX1* genes had indicated

the importance of a GTAC core in the CuRE (Quinn et al., 2000), the same motif that had been shown to be bound by the *Arabidopsis SPL* genes (Birkenbihl et al., 2005).
Crr1 contains motifs found also in the large SPL proteins of *Arabidopsis*. An AHA motif that was shown to be important for transcriptional activation (Kotak et al., 2004) is found in SPL1, SPL7, SPL12, SPL14 and SPL16. Ankyrin repeats, generally known to mediate protein-protein interaction (Sedgwick and Smerdon, 1999) are present in Crr1 and are also found in SPL1, SPL12, SPL14 and SPL16. As described above, one of the most obvious phenotypic alterations of the *spl1 spl12* double mutant compared to wild-type plants was a reduction in fertility. The finding that a SBP-box gene in *Clamydomonas* activates copper response genes, is particularly interesting to note since it has been shown that copper deficiency in *Arabidopsis* results in male sterility provoked by pollen defects (Jewell et al, 1988; Azouaou and Souvré, 1993). It is therefore tempting to speculate that *SPL1* and *SPL12*, like Crr1 in *Chlamydomonas* have an important function in controlling copper homeostasis in *Arabidopsis*. *SPL1* as well as *SPL12* are expressed rather constitutively (Cardon et al., 1999, Schmid et al., 2005) and hence could sense copper concentration throughout the whole plant life cycle. Like proposed for Crr1 in *Chlamydomonas* (Kropat et al., 2005), SPL1 and SPL12 could be in an inactive state on the presence of copper. In case of copper deficiency, the proteins may undergo a conformational change and become able to activate target genes necessary for copper repletion of the cell.

4.2.2 *SPL15* and *SPL9* may have partially redundant functions in controlling the plastochron and apical dominance

In contrast to *SPL1* and *SPL12*, which both are members of the large-sized *SPL* gene subfamily, *SPL9* as well as *SPL15* belong to the middle-sized SBP-box genes in *Arabidopsis* and are among the 11 members that have a recognition site for the miRNAs156 and 157.

4.2.2.1 Day-length specific functions of *SPL15*

The analysis of two *spl15* mutant allele's revealed phenotypic alterations compared to wild-type plants predominantly visible in short days. Under these conditions, two obvious phenotypic changes distinguish the mutant plants from wild type. First of all, *spl15* plants appear to bolt and to flower later than wild type. Second, *spl15* mutants lack the prophylls at the basis of the coinflorescences. In wild type these leaves were observed only under short day conditions and mainly at coinflorescences of the lower part of the stem. However, it remains unclear how these leaves are formed and why they are not present in long day grown plants.

In particular the delay in flowering under short day shown by the mutant immediately suggested a role of gibberellins (GAs) because a decrease in GA levels or insensitivity to GA signaling has been shown to delay flowering mainly in short day (Wilson et al., 1992). Exogenous GA application (data not shown) resulted for both, *spl15* and wild-type plants in earlier flowering as compared to non treated control plants. However, the mutants still showed the same relative delay to wild type as it was observed in untreated plants. The results clearly showed that the *spl15* mutants are still capable in sensing GA and also in mediating the GA signal to downstream target genes. Another possible explanation for the delayed transition may be improper cell division or cell elongation at the shoot such that the stem can not properly extend after the switch to reproductive growth. Cell division and cell elongation has been described as being downstream of auxin signaling (Leyser, 2001). Auxin action could also explain the growth of prophylls only at the lower part of the main inflorescence. It is well known that auxin is produced mainly in young tissue at the tip of the inflorescence and transported to more basal parts of the plant from there (Booker et al., 2003, Ljung et al., 2001). An auxin gradient could therefore be established with high concentrations at the tip of the inflorescence and low concentrations at the base. Formation of prophylls might require a certain auxin concentration that is established rather at the lower part of the main stem.

4.2.2.2 *SPL15*'s role on initiation of shoot meristem-derived lateral organs

Spl15 mutants develop many more rosette leaves than wild-type plants in short day, obviously due to an increased vegetative life span. However, careful analysis revealed that the *spl15* mutant plants also have a higher initiation rate of rosette leaves. This increased number of rosette leaves in *spl15* compared to wild type is therefore a result of both, a prolonged vegetative phase and in addition a shorter plastochron. In long day, *spl15* mutants also develop more rosette leaves than wild type. Under these conditions bolting and flowering time in the mutants does not differ from wild-type plants and the increased number of rosette leaves is thus entirely caused by a shortened plastochron.
The phenotypic observations suggest a function for *SPL15* in repressing lateral organ initiation. This assumption positively correlates with the spatial and temporal expression domain of *SPL15*. Global expression analysis of many developmental stages of *Arabidopsis* revealed strong expression of *SPL15* in the shoot apex (Schmid et al., 2005). Interestingly, *SPL15* expression in the shoot apex increased strongly after floral induction (Schmid et al., 2003). Additional support to the array data was given by a reporter construct showing strong *pSPL15*-driven GUS signal in the shoot apex. Surprisingly, the GUS signal appeared already in the shoot apical meristems as early as in the bend stage of the embryo.

4.2.2.3 *SPL15*'s role on the development of lateral roots

In addition to the shoot apex the GUS reporter construct showed a strong signal in the root apical meristem from the bend stage embryo onwards throughout the whole plant life. Later in root development the GUS signal also appeared at lateral root primordia and in the meristems of mature lateral roots. Further evidence that SPL15 is expressed in the root came from global expression data provided by Schmid and co-workers (Schmid et al., 2005).

Compared with the shoot apical meristem, the root apical meristem (RAM) has a simpler structure and fewer cells. At the heart of the RAM, the initial cells are arranged around a few mitotically inactive cells – the quiescent centre (QC).The QC appears to play a very important role in the maintenance of RAM activity. It retains the identity of the surrounding cells by inhibiting their differentiation (Nakajiama et al., 2002).
Two main inputs are important for the initiation and maintenance of this stem-cell niche: the GRAS family transcription factor SCARECROW (SCR) and auxin. Like the initiation of leaf primordia at the flanks of the SAM, the beginning of lateral root development is preceded by the accumulation of auxin (Malamy and Benfey, 1997). Again this increase in auxin concentration is mediated through the action of PIN family auxin efflux carriers.
Lateral root primordia (LRPs) arise from a subset of cells in the pericycle, termed pericycle founder cells, which are adjacent to the two xylem poles (Casimiro et al., 2003; Vanneste et al., 2005). After a series of transverse and periclinal divisions, cells undergo noticeable expansion and finally emerge from the parent root.

4.2.2.4 Possible role for *SPL15* in suppressing cell division or cell expansion

Taken together, the mutant phenotype and analysis of the expression data strongly suggest an important role for *SPL15* in tissues with high rates of cell division or cell expansion. *SPL15* expression according to the GUS signal could be detected in the radicle of the heart staged embryo, in the shoot as well as the root apical meristem, in lateral root primordia and in the style of the gynoceum. Interestingly the SPL15 expression domains overlap in large parts with regions of high auxin concentration (Benkova et al., 2003; Hu et al., 2003; Blilou et al., 2005). The plant hormone auxin plays an essential role in a wide range of plant growth and developmental processes, such as shoot and root formation and apical dominance (Davies, 1995). At the cellular level, auxin acts as a signal for cell division, expansion and differentiation (Leyser, 2001). As one possible explanation for the function of *SPL15* one could propose a role in auxin controlled cell division or cell expansion. Since the *spl15* mutant plants develop more shoot and root derived lateral organs, this role lies most likely in the repression of cell division and cell expansion.

4.2.2.5 Function of *SPL9* in apical dominance and the initiation of lateral organs at the shoot apical meristem

In contrast to *spl15* mutants, *spl9* plants did not show any effect on flowering time, neither under long day nor under short day condition. Nonetheless, in both conditions *spl9* mutants developed more rosette leaves than wild-type plants. The increase in the number of rosette leaves in *spl9* mutant plants was clearly due to a shorter plastochron. Flowering time was not affected and s*pl9* mutants developed about three more leaves than wild type before the first trichomes at the underside of the leaf appeared. Apart from a faster initiation of rosette leaves, apical dominance in the *spl9* mutant was reduced. Compared to wild type, more secondary shoots grew out at an earlier time point in development which resulted in a somewhat bushy phenotype (Unte, 2001).

Similar to *SPL15*, Schmid and co-workers could show a strong *SPL9* expression in the shoot apex and a quite strong induction upon floral transition (Schmid et al., 2003, 2005). According to global expression profiling, low *SPL9* expression could also be detected in rosette leaves (Schmid et al., 2005).

Analysis of the *spl9* mutant phenotype suggests a role of *SPL9* in repressing the initiation of rosette leaves at the shoot apex and in positively regulating apical dominance. Like the initiation of lateral organs, loss of apical dominance has shown to be regulated by the plant hormone auxin (Booker et al., 2003; Leyser, 2003). Auxin produced in the main shoot tip is believed to be the main repressor of axillary bud outgrowth (Schmitz and Theres, 2005; Beveridge, 2006). Thus *SPL9* could be positively acting on the repression of lateral bud outgrowth by transmitting auxin signals.

4.2.2.6 *SPL9* and *SPL15* carry out partially redundant functions at the shoot apex

As mentioned before, *SPL9* and *SPL15* belong to the middle-sized *SPL* genes and have both a recognition site for the miRNAs156 and 157 in the coding region of their last exon. *SPL9* and *SPL15* are more closely related to each other than to any other of the *Arabidopsis SPL* genes and might well represent paralogous genes. Their expression domains overlap spatially and temporally, at least for the shoot apex where both genes appear to control the initiation of lateral organs. To uncover potential functional

redundancy, a *spl9 spl15* double knock-out mutant was created and analyzed subsequently.

Both *spl15* and *spl9* single mutant plants developed rosette leaves faster than wild-type plants. Interestingly, mutation of both genes simultaneously resulted in a plastochron shorter than that of either single mutant. In both long and short day conditions the double mutant developed substantially more rosette leaves than both of the single mutants. In addition trichomes at the abaxial side appeared at a much later rosette leaf than it was observed for the single mutants. Loss of apical dominance discussed for the *spl9* single mutant, appeared to be enhanced in the *spl9 spl15* double knock-out.

The delay in flowering under short day condition and the lack of prophylls, both characteristic features of the *spl15* single mutant did not become more severe in the *spl9 spl15* double knock out.

In summary it can be said, that the phenotypic alterations caused by the loss of *SPL9* function (i.e. shorter plastochron, loss of apical dominance) became more severe upon loss of both, *SPL9* and *SPL15* function. In contrast, phenotypic changes as a result of the loss of *SPL15* function (i.e. later bolting in short day, lack of prophylls in short day) did not become stronger in the double knock out.

The additive effects of *spl9* and *spl15* with respect to plastochron and apical dominance led to the assumption that both genes act together in repressing the initiation of lateral organs and the outgrowth of axillary buds at the shoot.

Until now there are only very few mutants known that exhibit a shortened plastochron. In maize, *terminal ear1* (*te1*) mutants show enhanced leaf production and aberrant phyllotaxis (Veit et al., 1998). *Arabidopsis altered meristem program 1* (*amp1*) was also reported to have an altered plastochron and phyllotaxis (Helliwell et al., 2001). These mutants are defective in both plastochron and phyllotaxis. The pleiotropic defects make it difficult to interpret the gene function explicitly.

So far, the only mutants that specifically affect the plastochron but not the phyllotaxis were found in rice. Here, the cytochrome P450 encoding *PLA1* gene was shown to regulate the rate of leaf initiation and the time of the vegetative to reproductive phase change (Miyoshi et al., 2004). Mutations in the *PLA2 gene,* a MEI2-like RNA binding

protein encoding gene, were reported to exhibit a shortened plastochron, reduction of leaf size and the conversion of primary reproductive branches into vegetative shoots (Kawakatsu et al., 2006). Interestingly, similar to *spl15* mutants both *pla1* and *pla2* plants were reported to have a delayed transition to reproductive phase, suggesting a similar biological function.

Kawakatsu and co-workers suggest that the plastochron is more closely correlated to a higher rate of cell divisions than to a larger SAM size because despite the fact that the SAM of the *pla2* mutant is smaller than that of the *pla1* mutant, it has a shorter plastochron. Similarly, the double mutant *pla1 pla2* has a smaller SAM than either of the single mutants, nevertheless it has the shortest plastochron (Kawakatsu et al., 2006).

Kawakatsu et al. proposed a model in which preexisting leaf primordia inhibit the precocious initiation of the next leaf. Leaves loose this inhibitory effect as they grow older and as a consequence the next leaf can be initiated. In *pla2* mutants of rice, leaves mature faster and therefore the inhibitory effect declines more rapidly, resulting in more rapid leaf initiation (Kawakatsu et al., 2006). A similar model could be proposed for *SPL9* and *SPL15* (Figure 4.1.). For example, both genes repress maturation of lateral organs at the SAM in inhibiting cell division or cell expansion. Thus, they inhibit the initiation of the next primordium. In *spl9* and *spl15* mutants however maturation of lateral organs appears faster and the inhibiting signal for the next primordium is cancelled sooner. Interestingly, overexpressing of both, *SPL9* and *SPL15* led to less and narrower rosette leaves, suggesting that overexpression of both genes "overrepress" the maturation of lateral organs and the time for the signal inhibiting the initiation of the next primordium is prolonged. The narrow shape of the leaves could be explained by an altered cell expansion or cell division. An interesting aspect of the proposed model can be deduced from the fact that the expression of *SPL15* and *SPL9* as well as of other miRNA regulated *SPL* genes is strongly induced upon floral induction (Schmid et al., 2003). At the same time the expression level of the miRNA156 decreases (Schwab et al., 2005). According to the model, higher expression level of *SPL15* and *SPL9* would lead to an increased inhibition of leaf development. Since less stem cells have to be recruited to form new leaf primordia, more cells are available to form the extending stem after bolting.

Spl15 mutant plants not only developed more rosette leaves, they also were shown to develop more lateral roots. Thus a similar model than the one suggested for the initiation of shoot derived lateral organs could be applied onto the root. Already formed lateral root primordia (LRPs) inhibit the initiation of new primordia. During maturation, this signal becomes weakened and eventually allows the formation of a new LRP. *SPL15* represses the maturation of lateral roots and by doing so, prolongs the signal inhibiting the initiation of new LRPs (Figure 4.3.).

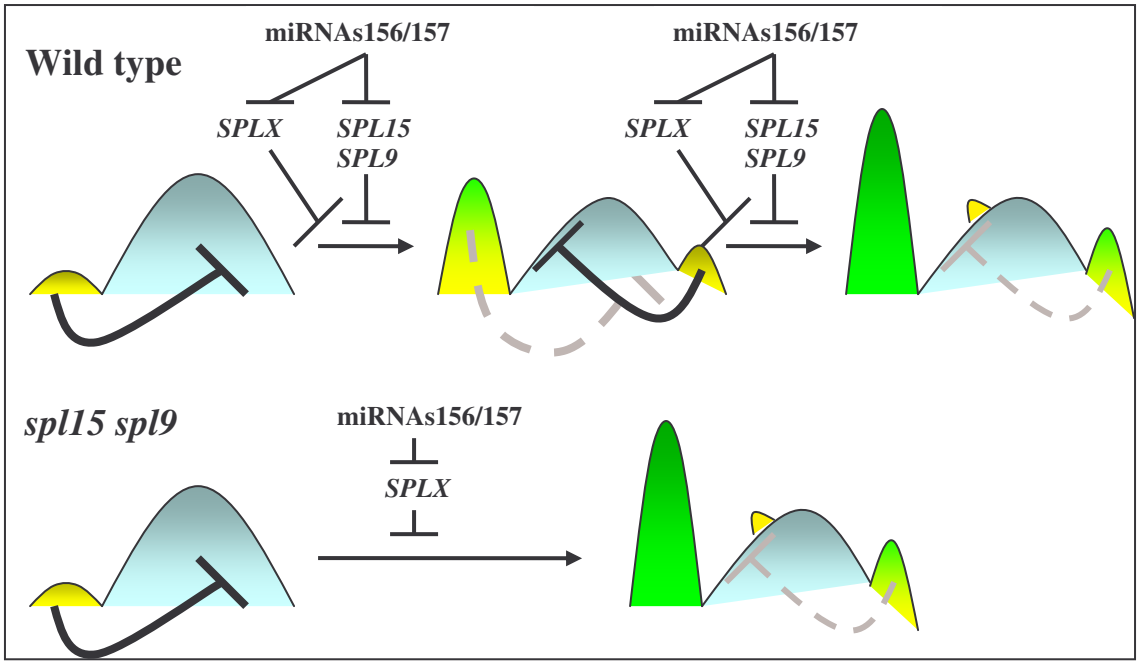

Figure 4.1.: Model of plastochron regulation adapted from Kawakatsu et al., 2006.
The initiation of new leaves is inhibited by preexisting premature leaf primordia. *SPL15* as well as *SPL9* slows down the rate of leaf development for example by repressing cell division or cell expansion. The inhibitory effect becomes weakened as leaf development proceeds, finally allowing the next leaf to be initiated. In the *spl15*, *spl9*, the *spl9 spl15* double mutant and in the miRNA overexpressor, leaves develop faster and the inhibitory effect is cancelled sooner than in wild type.

4.2.3 Other *SPL* genes may also be involved in controlling the plastochron and apical dominance

Schwab and co-workers showed that overexpression of the miRNA156b locus results in down-regulation of all of the *SPL* genes represented on the ATH1 chip that have a miRNA recognition site in their last exon (Schwab et al., 2003). Interestingly, phenotypic analysis of these transgenic plants revealed a shorter plastochron and a more severe loss of apical dominance than that of the *spl9spl15* double mutant, indicating that other *SPL*

genes are participating in the regulation of the plastochron and axillary bud outgrowth in *Arabidopsis* as well. It would be interesting to know whether successively knocking out more *SPL* genes would result in a successively more severe phenotype. However, first results obtained from *spl2spl9spl15* triple mutants (data not shown) indicate that this is not the case because the leaf initiation rate of the triple mutant appeared to be similar to that of the double mutant.

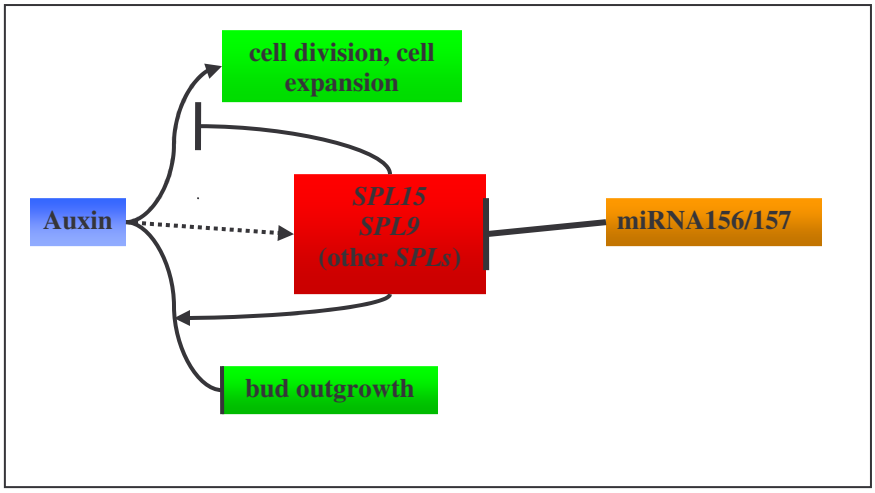

Figure 4.2.: Working model of *SPL9* and *SPL15*.
SPL15 and *SPL9* and possibly other *SPL* genes positively regulate the auxin mediated repression of axillary bud outgrowth. At the same time they repress the auxin derived signal on cell division and cell expansion. *SPL15* and *SPL9* are negatively controlled by the miRNAs156 and 157.

Probably not all of the eleven miRNA controlled *SPL* genes are responsible for the regulation of the lateral organ initiation rate.

As discussed before, although evolutionary closely related, *SPL9* and *SPL15* do not fulfill completely redundant functions. For some reason however, the expression of both genes together with eight other *SPL* genes in *Arabidopsis* is under a common tight regulatory mechanism based on a miRNA. It will be of particular interest in the future to get an answer to the questions what the general function of those genes is and why they have to be controlled together.

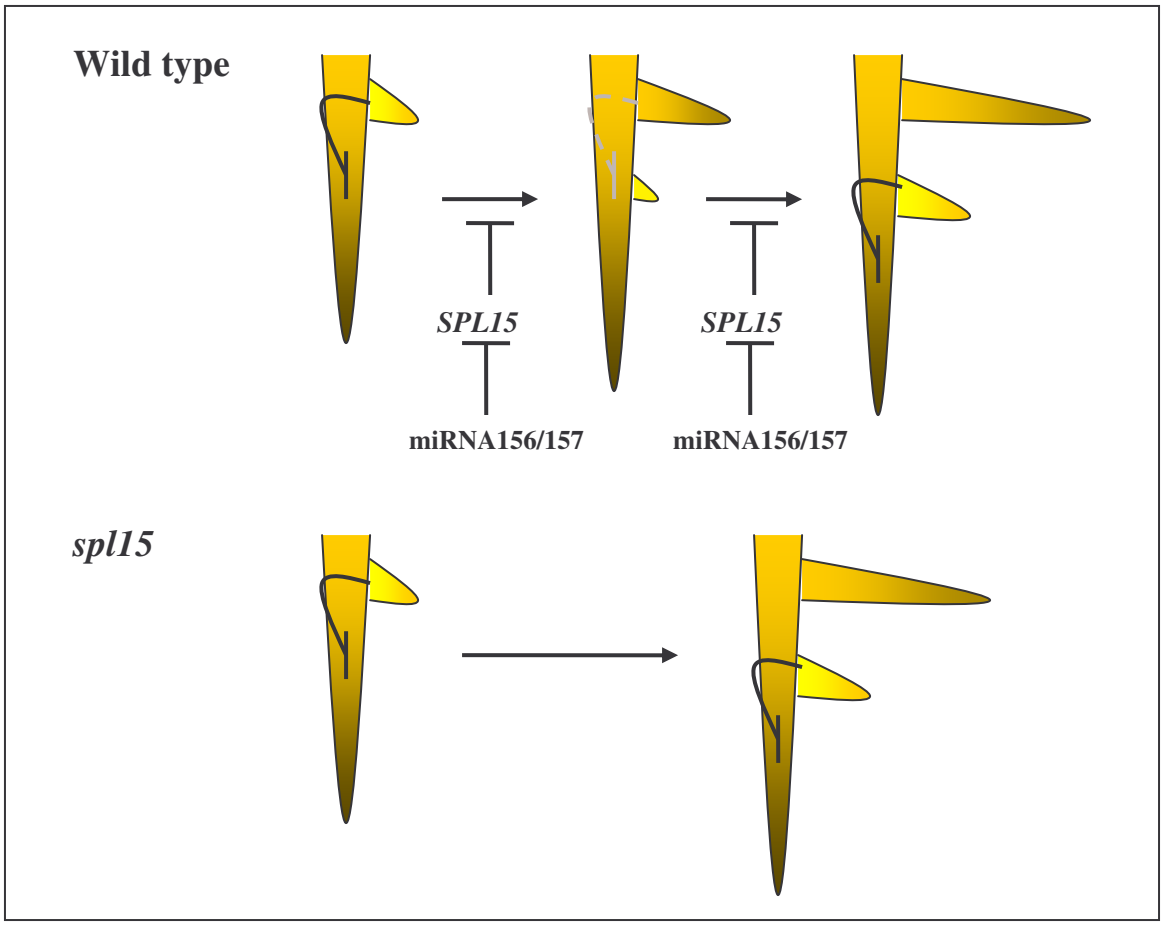

Figure 4.3.: Model on regulation of lateral root growth.
Similar to the model described for the regulation of the plastochron. Initiation of new lateral roots is inhibited by preexisting premature lateral root primordia. *SPL15* slows down maturation of the root. The inhibitory effect becomes weakened as lateral root development proceeds, finally allowing a new lateral root primordium to be initiated. In the *spl15* mutant maturation of lateral roots proceeds faster leading to a sooner cancellation of the inhibiting signal and a faster initiation of the next primordium.

4.3 Conclusions and future perspectives

Here, the identification of potential target genes of the *Arabidopsis* transcription factor SPL8 is reported. By means of global expression analysis putative *SPL8* target genes could be identified. Analysis of the target gene promoter regions revealed in addition that the previously described SPL8 DNA-binding motif GTAC is overrepresented in the target genes as compared to a random promoter set. For many of the identified genes a function in anther development could be suggested. However, future research will still be necessary to gain more detailed information on the identified SPL8 targets in order to help understanding anther development in plants. For example, plants mutated in the

putative target genes should be analyzed on phenotypic and molecular level. In addition, physical interaction of SPL8 with its target gene promoters could be analyzed with the help of the X-ChIP technology. Genetic analysis by crossing *spl8* plants with other known anther-defective mutants should further help to elucidate *SPL8* function.

Careful mutant analysis revealed that different members of the diverse SBP-box gene family in *Arabidopsis* appear to have diverse functions in multiple aspects of plant development. *SPL1* and *SPL12*, representing the large *SPL* genes in *Arabidopsis* could be shown to function in developing of the plant architecture and fertility. Both genes might be involved in the regulation of copper homeostasis as it has been shown for a evolutionary related gene in *Chlamydomonas* (Kropat et al., 2005).
SPL15 and *SPL9*, both representing the middle-sized *SPL* genes were shown to be mainly involved in the initiation of lateral organs at the shoot and in the root (*SPL15*). Furthermore both genes appear to be important in mediating apical dominance. A model for the initiation of shoot and root derived lateral organs suggests a function for *SPL9* and *SPL15* in slowing down maturation of lateral organs for example by repression of cell division or cell expansion.
In the future further mutants will have to be analyzed to get a better picture on whether all miRNA controlled *SPL* genes have similar biological functions. Furthermore, global expression analysis should reveal target genes that would help to elucidate regulatory pathways.

5 Abstract

In *Arabidopsis thaliana*, 17 plant specific transcription factors are known to be members of the *SQUAMOSA PROMOTER BINDING PROTEIN LIKE* (*SPL*) gene family. This family of *Arabidopsis* SBP-box genes can be divided into subfamilies based on their genomic organization and sequence similarities. *SPL1*, *SPL7*, *SPL12*, *SPL14* and *SPL16* form one subfamily representing the largest and most complex members. The second subfamily is formed by the mid-sized genes *SPL2*, *SPL6*, *SPL8*, *SPL9*, *SPL10*, *SPL11*, *SPL13a*, *SPL13b* and *SPL15*. Finally, the small genes *SPL3*, *SPL4* and *SPL5* represent the third subfamily within the *Arabidopsis SPL* gene family.

SBP-box genes have been exclusively found in plants and hence their functions were proposed to be plant specific. However, when the work of this thesis started, the role of *SPL8* in anther development was the only known function of a SBP-box gene in *Arabidopsis*. In order to gain more information about the role of the transcription factor *SPL8* within this developmental process, global expression analysis was used to identify target genes. Analysis of the target gene promoter regions showed an overrepresentation of the known SBP-box DNA binding motif GTAC, additionally supporting the significance of the identified genes.

Reverse genetics was used in order to find out more about the role of the other *SPL* genes in *Arabidopsis* development. The results of this work led to the assumption that the two paralogous genes *SPL1* and *SPL12* have redundant functions in controlling flowering time in long days, rosette leaf size and fertility.

Furthermore it could be shown that the two evolutionary probably very closely related genes *SPL9* and *SPL15* have redundant function in controlling apical dominance and the initiation rate of lateral organs at the shoot apex.

Based on the results gained in this study the *SPL* genes are very likely to be separated into functional subclasses in which the group of large genes has a distinct function from the miRNA regulated mid-sized and small genes. Within the *SPL* genes in *Arabidopsis*, *SPL8* seems to be an exception with respect to its phyllogenetic and functional affiliation.

6 Zusammenfassung

Die *SQUAMOSA PROMOTER BINDING PROTEIN LIKE (SPL)*-Genfamilie in *Arabidopsis thaliana* wird von 17 pflanzenspezifischen Transkriptionsfaktoren gebildet, die sich durch eine stark konservierte DNA-Bindedomäne auszeichnen; die SBP-Domäne. Die Mitglieder der *SPL*-Genfamilie können aufgrund ihrer Größe und phylogenetischer Ähnlichkeiten in drei verschiedene Unterfamilien aufgeteilt werden. *SPL1*, *SPL7*, *SPL12*, *SPL14* und *SPL16* gehören anhand dieser Kriterien zu den großen *SPL* Genen. Dagegen unterteilen sich *SPL3*, *SPL4* und *SPL5* in die kleinen und *SPL2* *SPL6*, *SPL8*, *SPL9*, *SPL10*, *SPL11* *SPL13a*, *SPL13b* und *SPL15* in die mittelgroßen Gene.

Die Beteiligung von *SPL8* an der Entwicklung der Antheren war bei Beginn dieser Arbeit die einzige biologische Funktion, die einem SBP-Box Gen in *Arabidopsis* zugeteilt werden konnte. Um einen genaueren Überblick darüber zu bekommen, welche Rolle der Transkriptionsfaktor *SPL8* innerhalb dieses Entwicklungsprozesses einnimmt, wurden mit Hilfe einer globalen Expressionsanalyse Zielgene identifiziert. Das DNA-Bindemotiv der SBP-Box Gene ist das Tetranukleotid GTAC. Eine Computer gestützte Analyse der Zielgen-Promotorregionen zeigte eine signifikante Häufung dieses Bindemotives im Vergleich mit Promotoren zufällig ausgewählter Gene und bestätigte damit die Bedeutsamkeit der identifizierten Zielgene.

Im weiteren Verlauf der Arbeit wurde mit Hilfe der „Reversen Genetik“ die mögliche Funktion weiterer *SPL* Gene aufgeklärt. Die beiden großen, paralogen Gene *SPL1* und *SPL12* zeigten dabei redundante Funktionen innerhalb der Kontrolle der Blütezeit, Blattgröße und Fertilität.

Weiterhin zeigen die Ergebnisse dieser Arbeit, dass die beiden mittelgroßen, paralogen *SPL* Gene *SPL9* und *SPL15* eine wichtige und teilweise redundante Funktion in der Kontrolle des Plastochrons sowie der apikalen Dominanz einnehmen.

Im Verlauf dieser Arbeit konnte gezeigt werden, dass die SBP-Box Gene in *Arabidopsis*, trotz ihrer phylogentischen Zugehörigkeit zu einer Familie, funktionelle Sub-Familien bilden. Wahrscheinlich bilden hierbei die großen *SPL* Gene eine funktionelle Gruppe.

Eine zweite Gruppe wird vermutlich durch die miRNA-regulierten kleinen und mittelgroßen Gene gebildet. *SPL8* nimmt hierbei innerhalb der *SPL* Gene eine Ausnahmeposition hinsichtlich seiner phylogenetischen und funktionellen Zugehörigkeit ein.

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Yamasaki, K., Kigawa, T., Inoue, M., Yamasaki, T, Yabuki, T., Aoki, M., Seki, E., Matsuda, T., Tomo, Y., Terada, T., Shirouzu, M., Tanaka, A., Seki, M., Shinozaki, K., Yokoyama, S. (2006). An *Arabidopsis* SBP-domain fragment with a disrupted C-terminal zinc-binding site retains its tertiary structure. FEBS Letters, Vol. 580, 2109-2116

Zhang, J. (2005). The SBP-Box Gene *SPL8* Affects Reproductive Development and Gibberellin Response in Arabidopsis. PhD Thesis, University of Cologne, Germany

8 Appendix

8.1 Appendix A: RT-PCR on SPL8 target genes

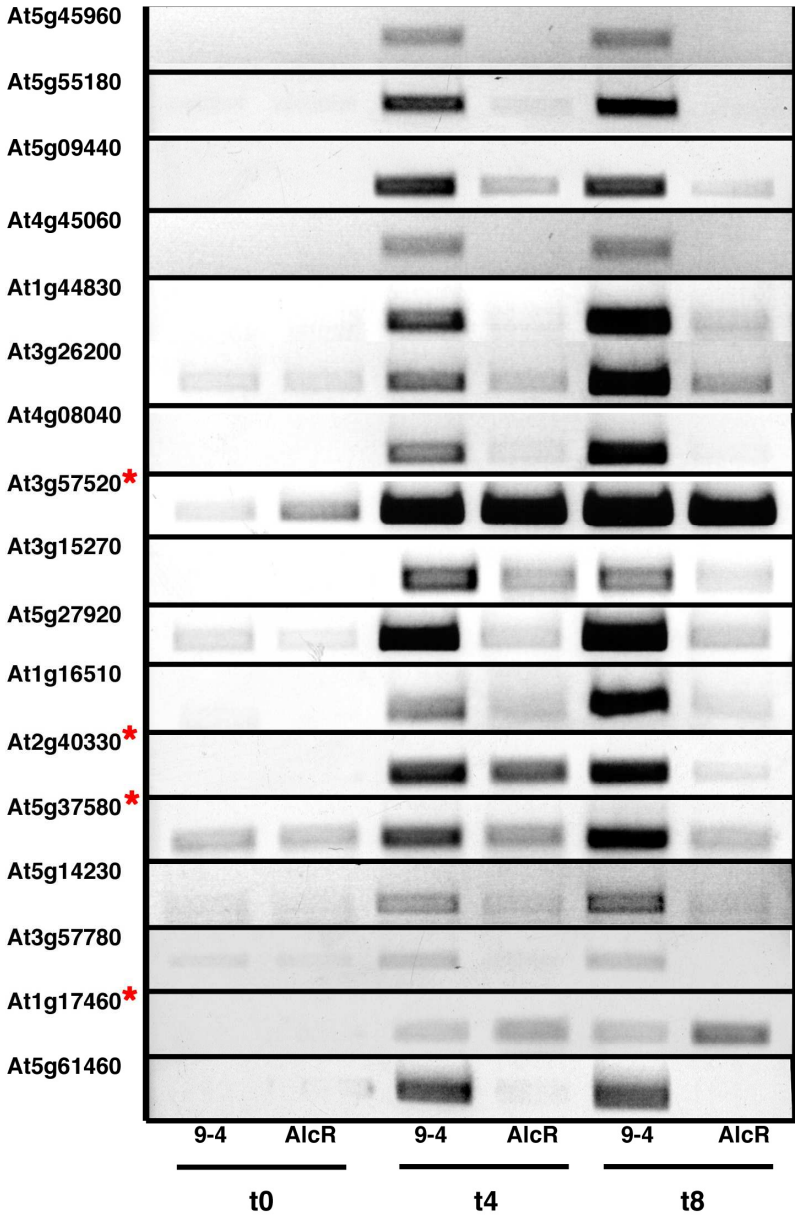


Figure A I: RT-PCR results of the 28 top candidates Figure legend on next page.

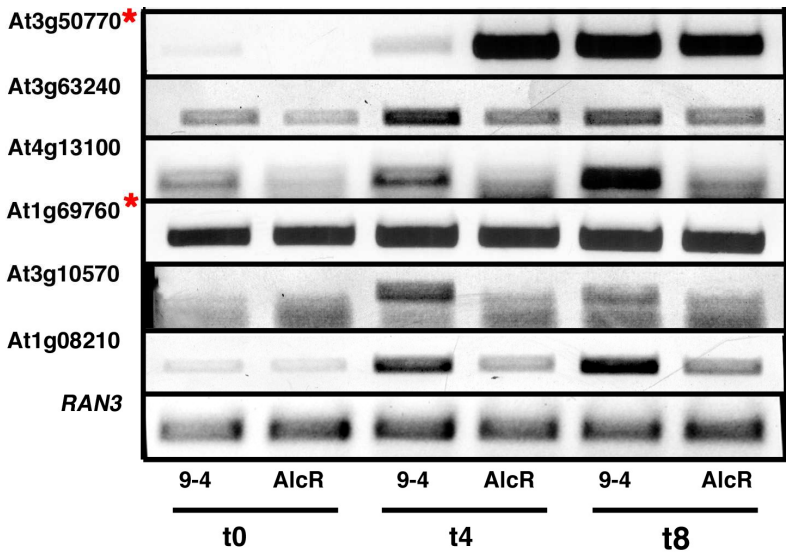
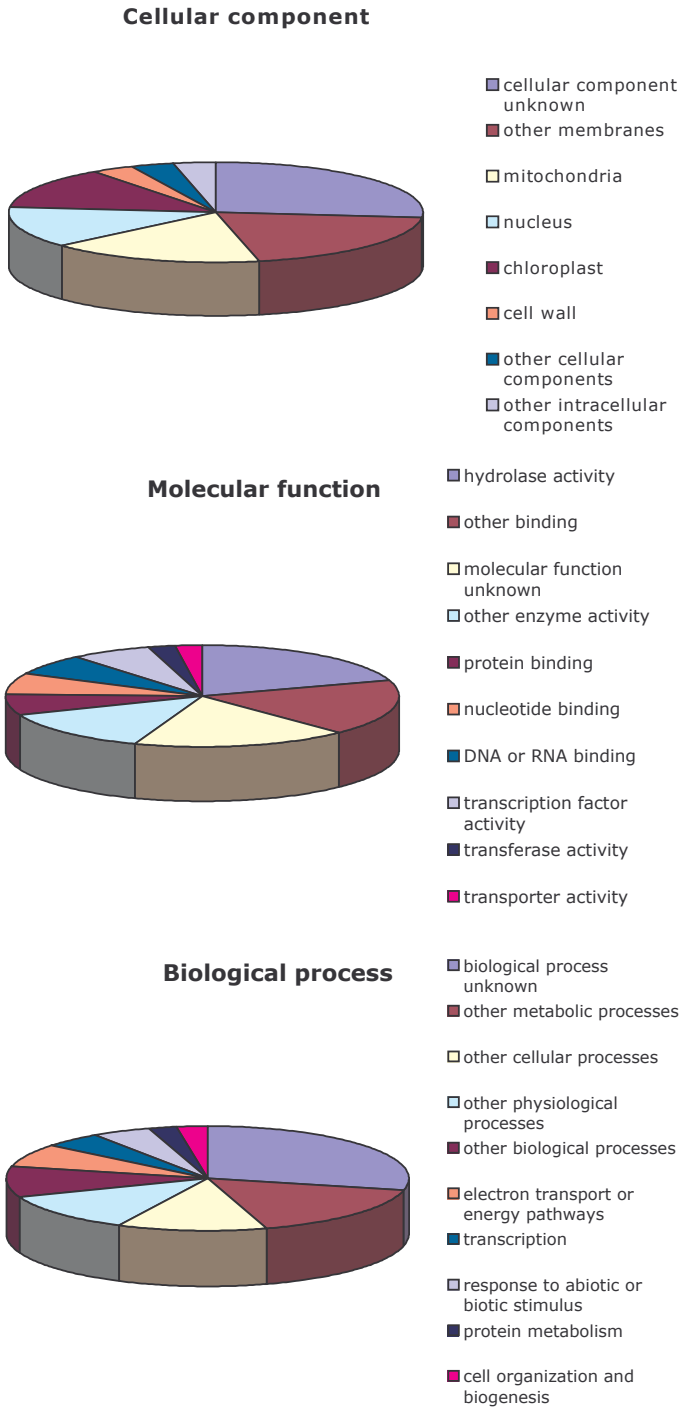


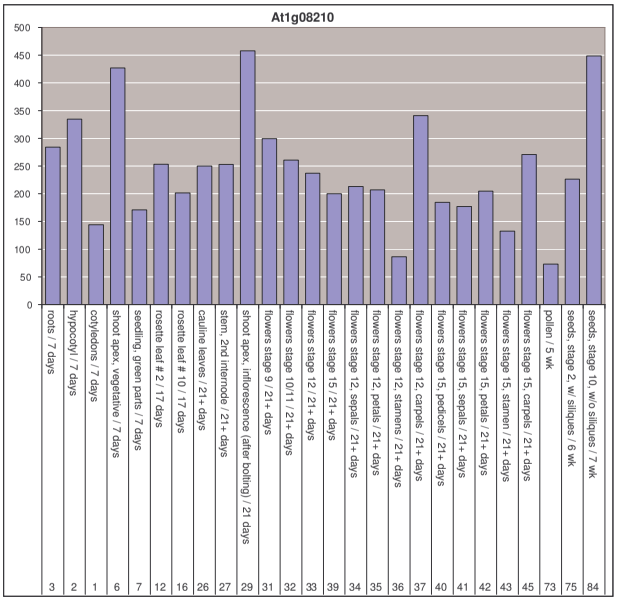
Figure A I: RT-PCR results of the 28 top candidates revealed the expression level before and four as well as eight hours after ethanol induction. The SPL8 inducible line 9-4 as well as a AlcR control plant was tested. Red stars indicate genes that showed differential expression in the micro-array experiment but appeared to be a false positive candidate according to the RT-PCR data.

8.2 Appendix B: Annotation of identified SPL8 target genes

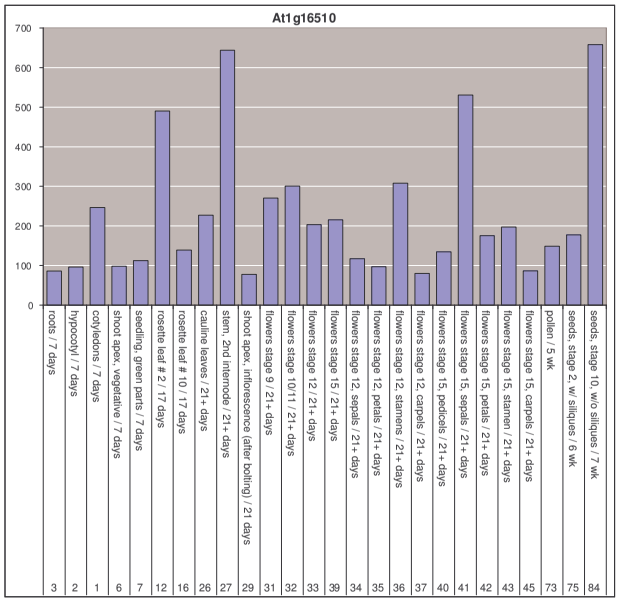


8.3 Appendix C: Expression levels of SPL8 target genes

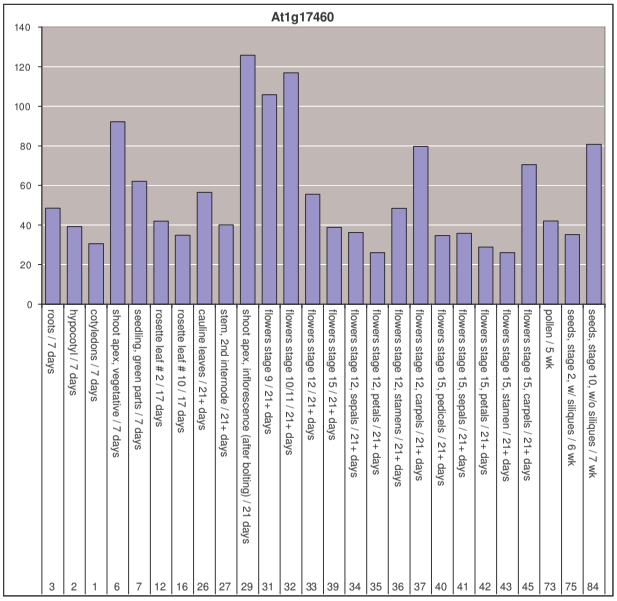
Expression levels of *SPL8* target genes over different developmental stages. (Data taken from AtGenexpress, Schmid et al., 2005).



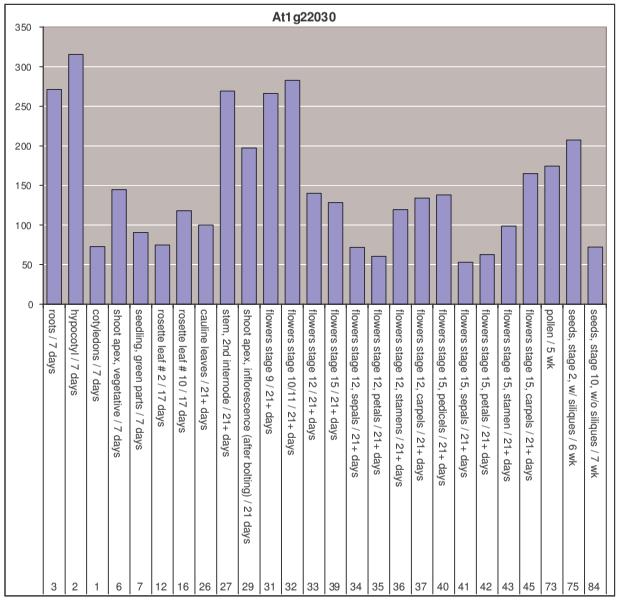
aspartic protease family protein



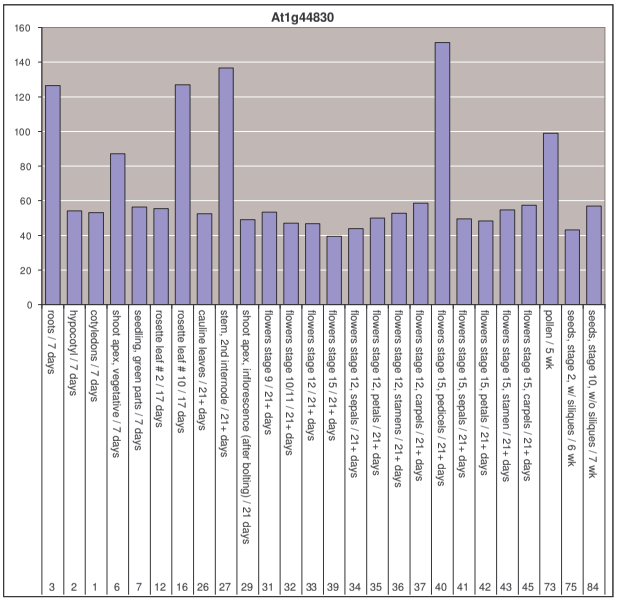
auxin-responsive family protein



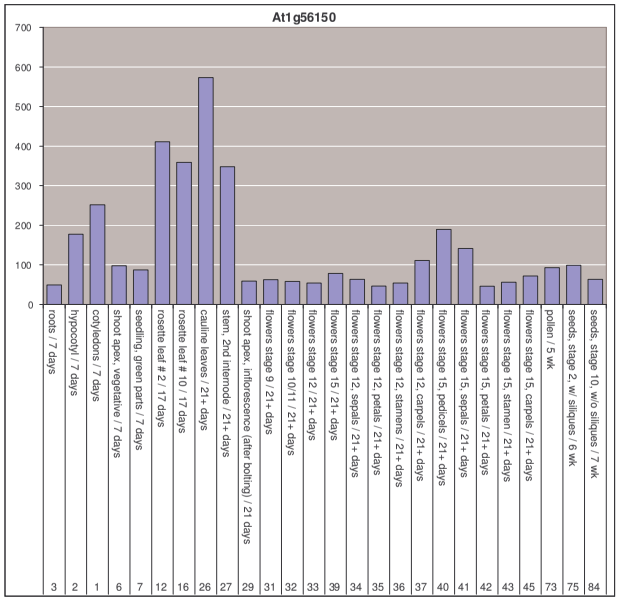
myb family transcription factor



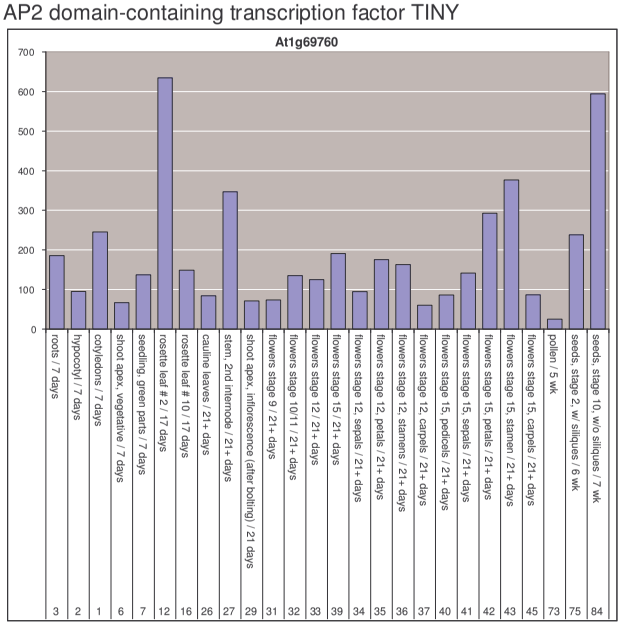
expressed protein



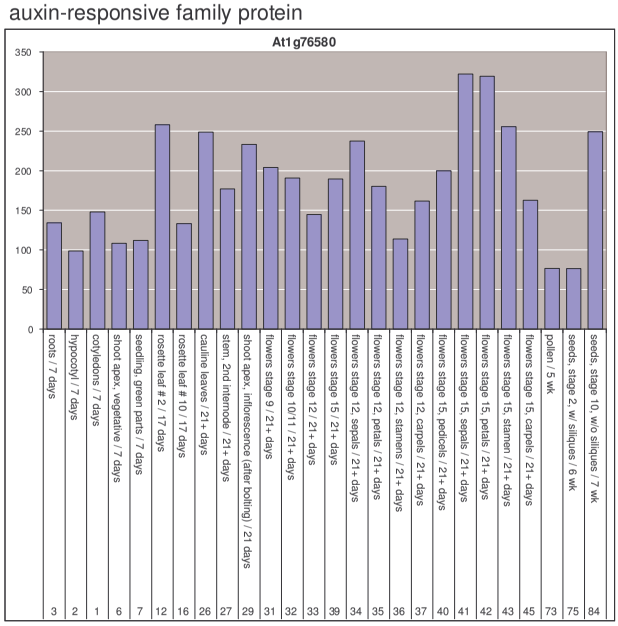
AP2 domain-containing transcription factor TINY



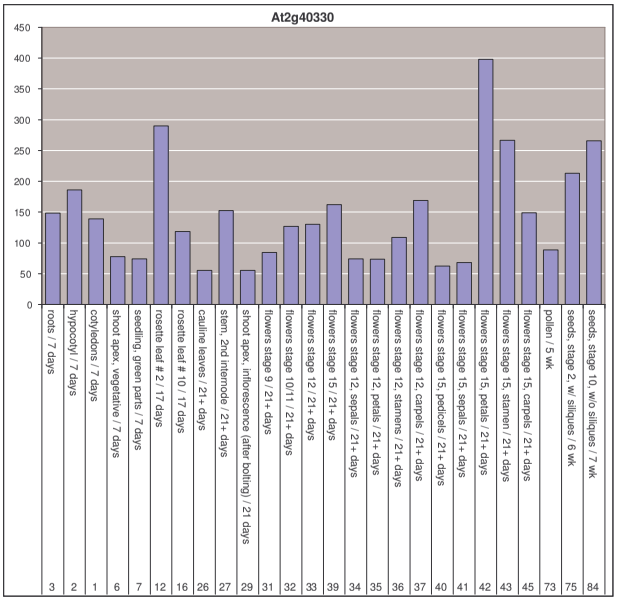
auxin-responsive family protein



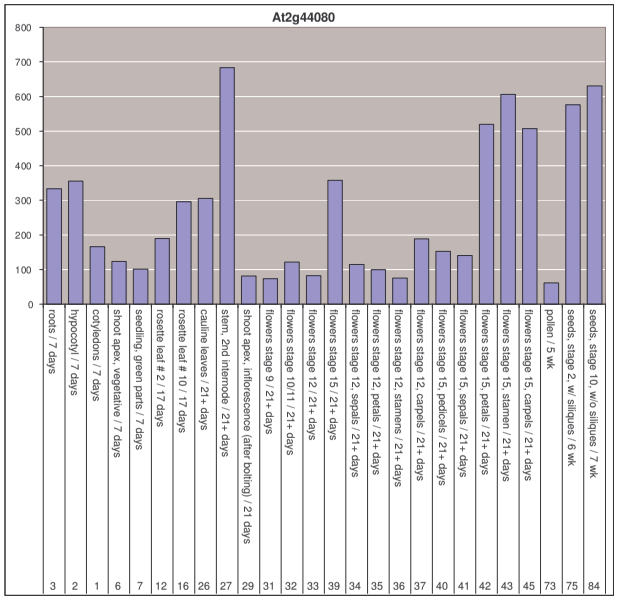
expressed protein



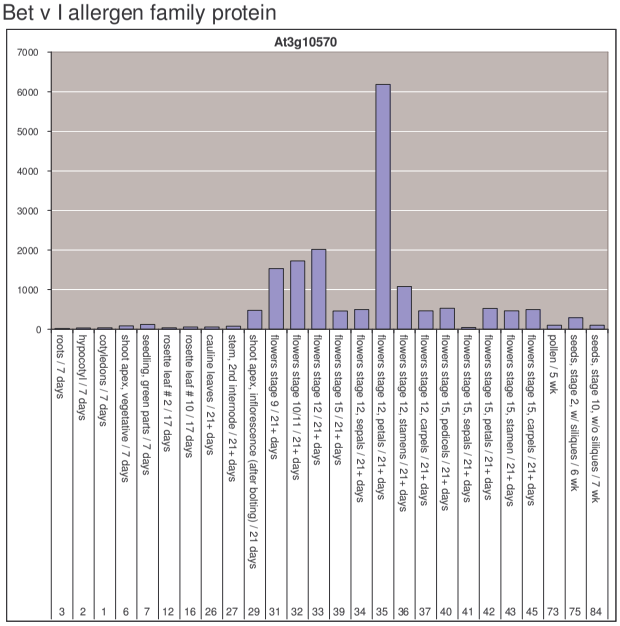
SPL1-Related3 protein (AtSPL16)



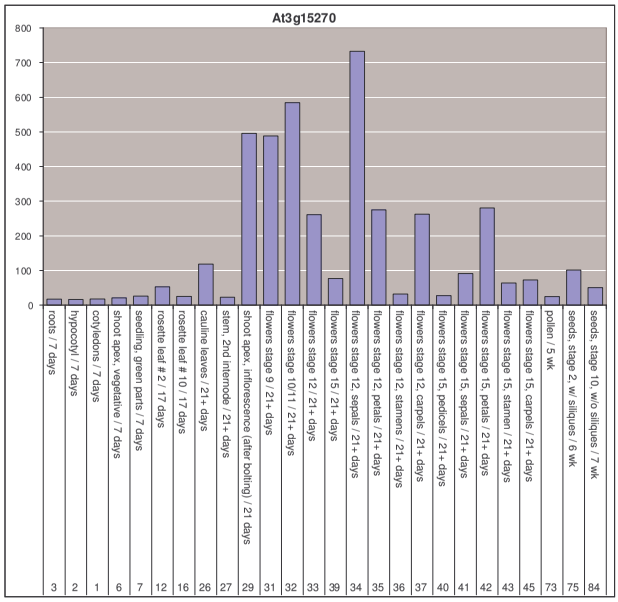
Bet v I allergen family protein



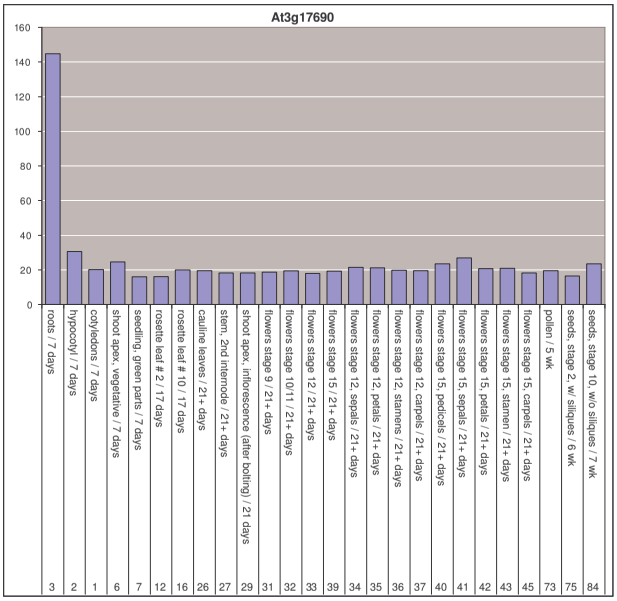
expressed protein



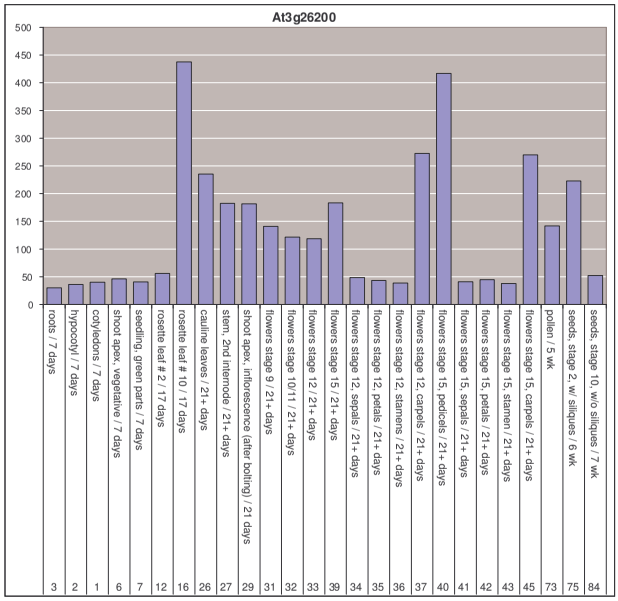
cytochrome P450



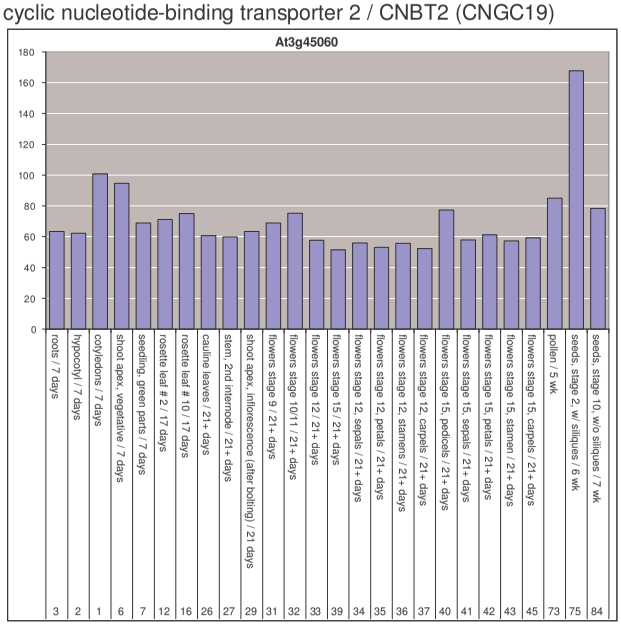
squamosa promoter-binding protein-like 5 (SPL5)



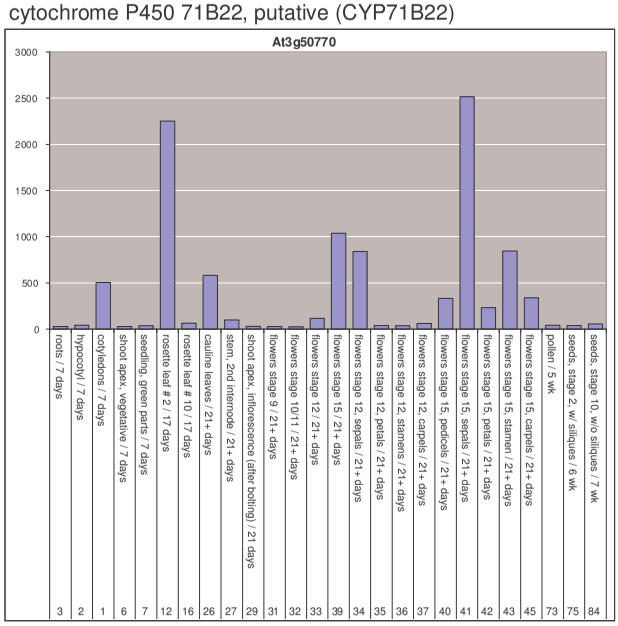
cyclic nucleotide-binding transporter 2 / CNBT2 (CNGC19)



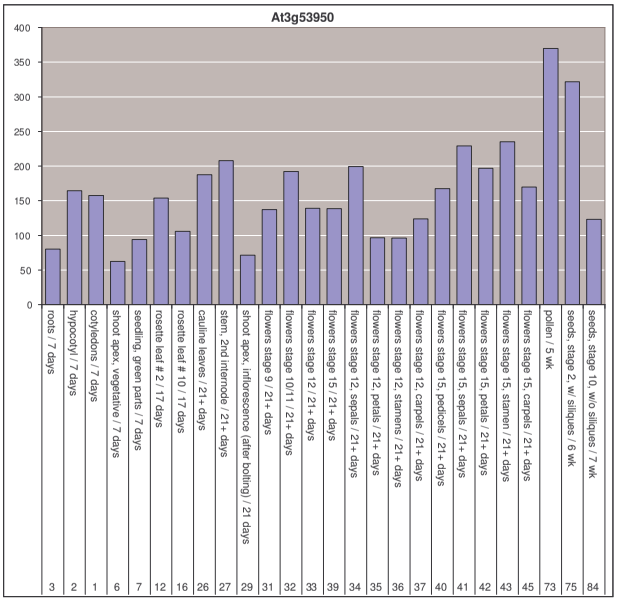
cytochrome P450 71B22, putative (CYP71B22)



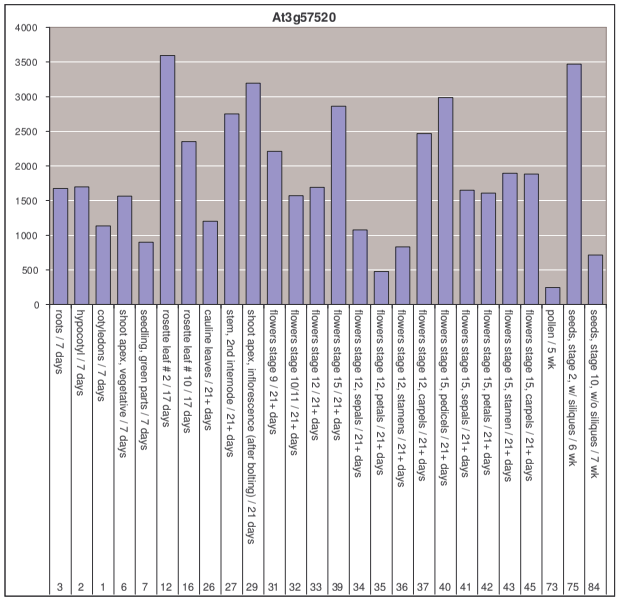
high-affinity nitrate transporter



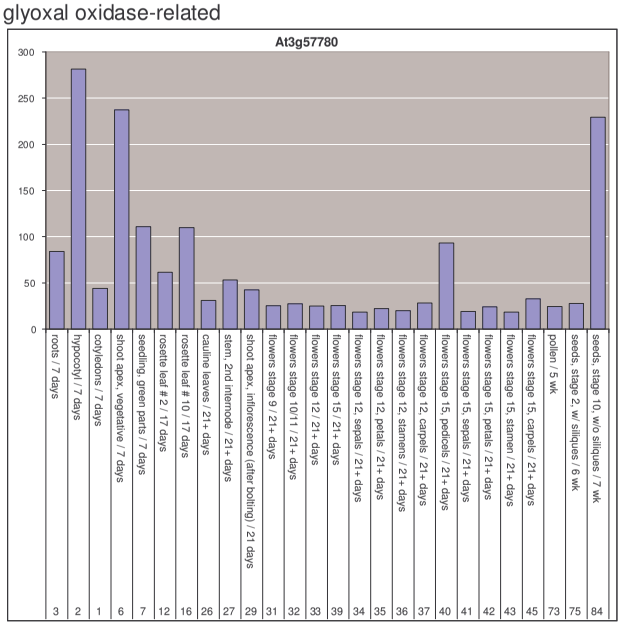
calmodulin-related protein, similar to regulator of gene silencing



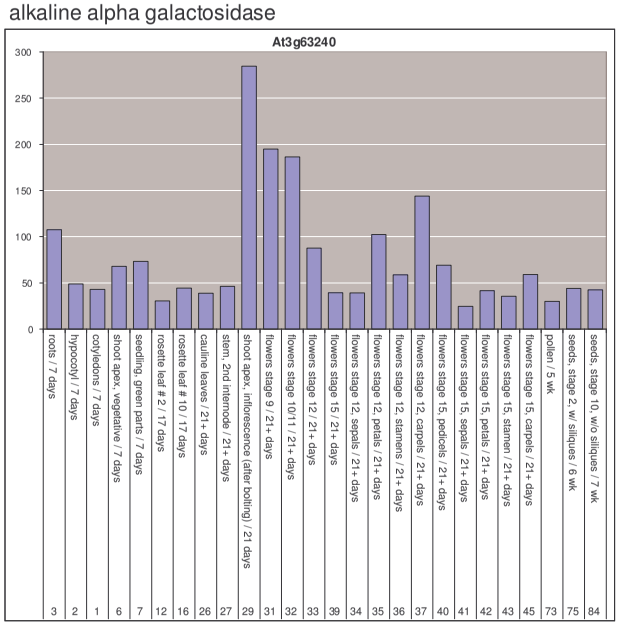
glyoxal oxidase-related



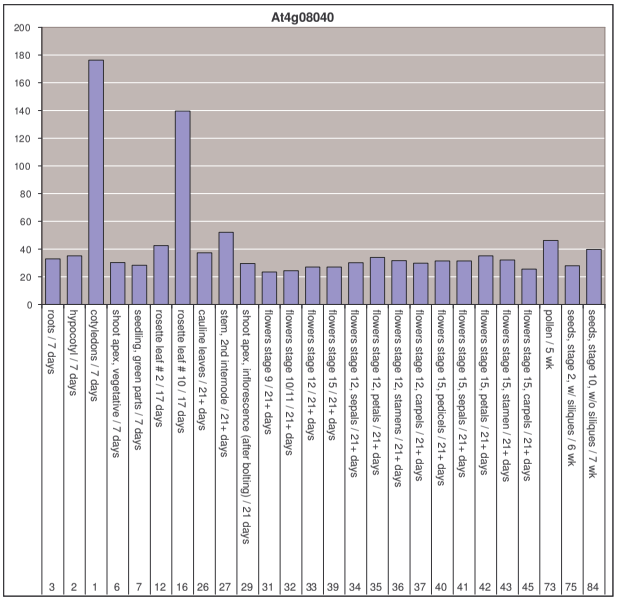
alkaline alpha galactosidase



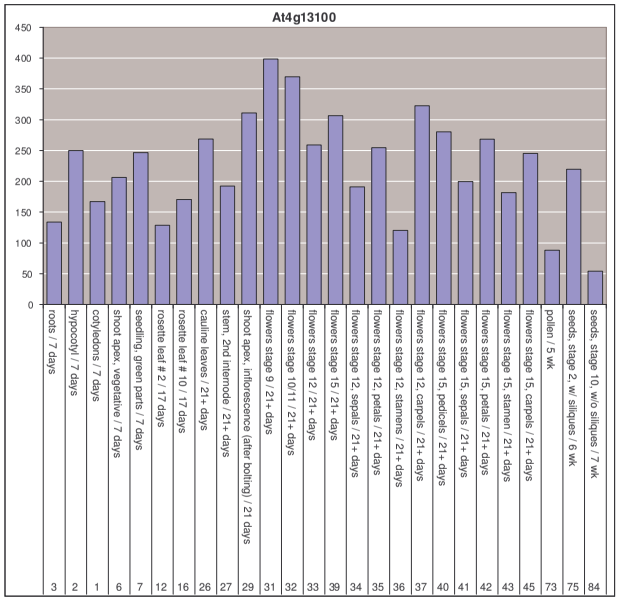
expressed protein



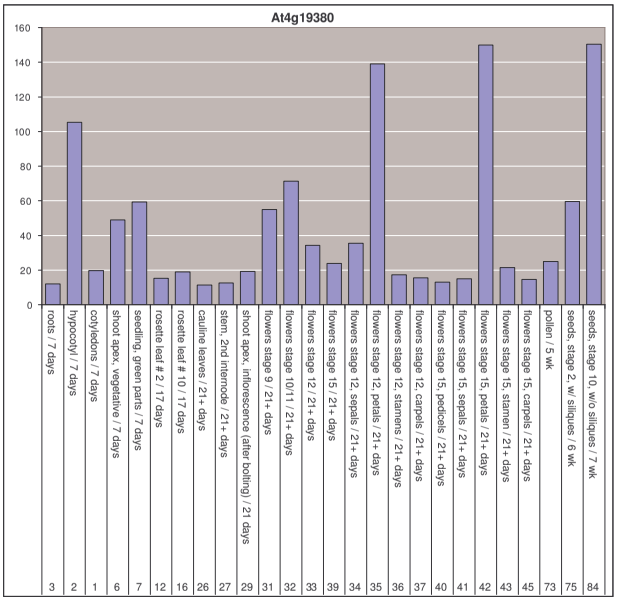
endonuclease/exonuclease/phosphatase family protein



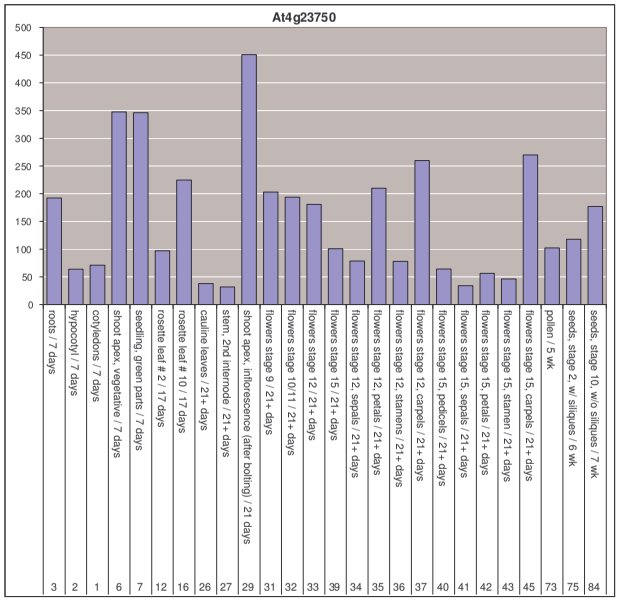
1-aminocyclopropane-1-carboxylate synthase, putative / ACC synthase



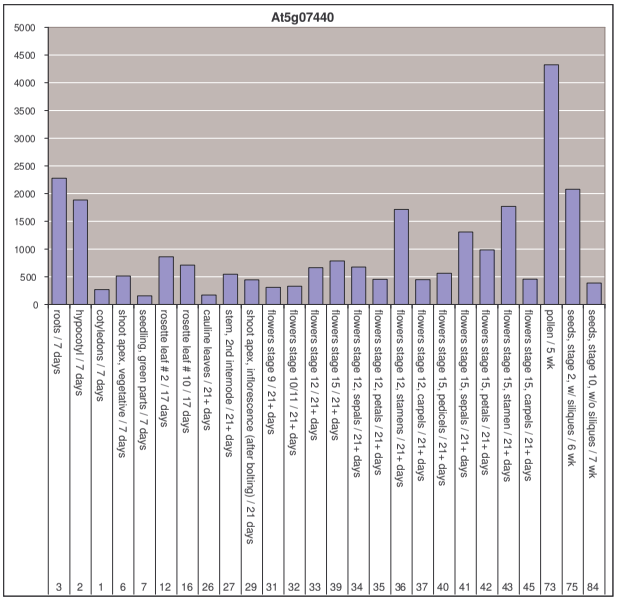
zinc finger (C3HC4-type RING finger) family protein



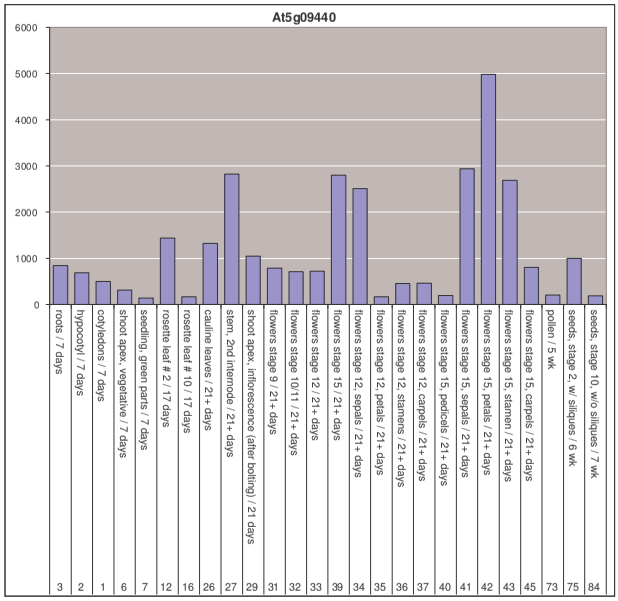
alcohol oxidase-related similar to long chain fatty alcohol oxidase



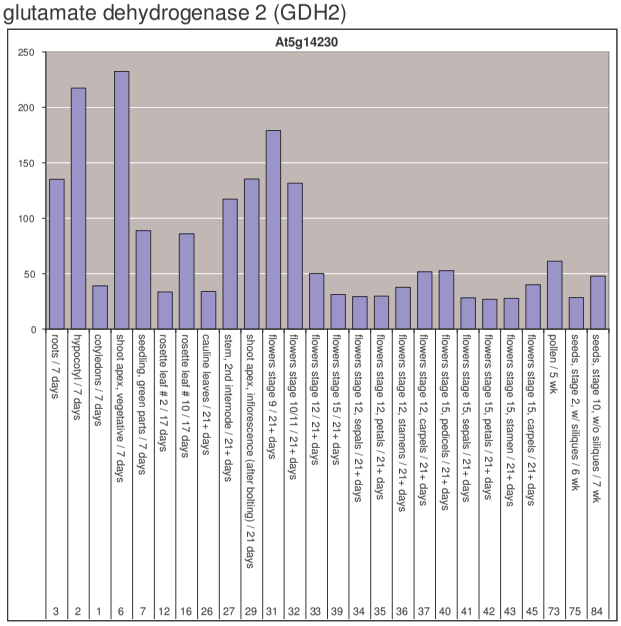
AP2 domain-containing transcription factor



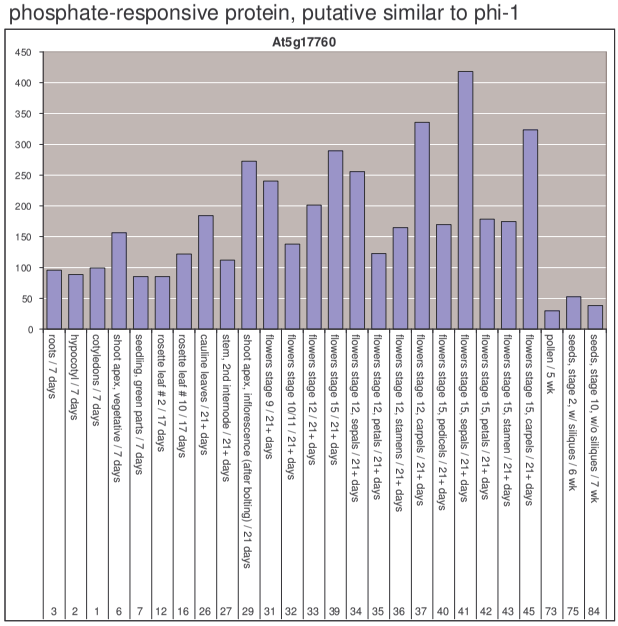
glutamate dehydrogenase 2 (GDH2)



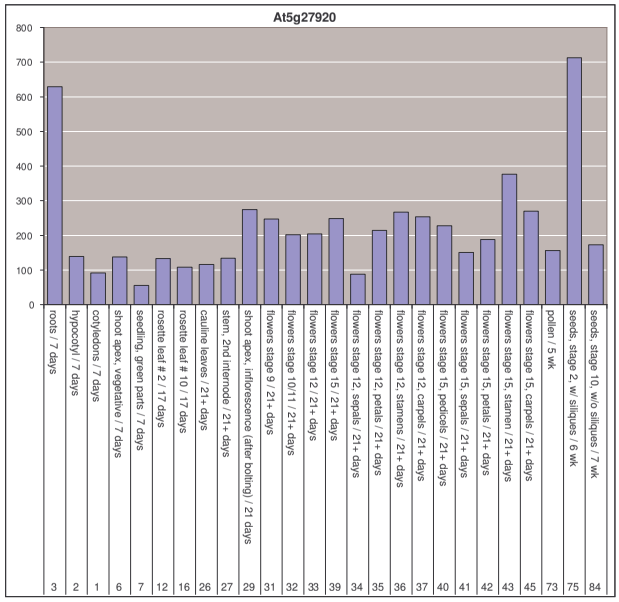
phosphate-responsive protein, putative similar to phi-1



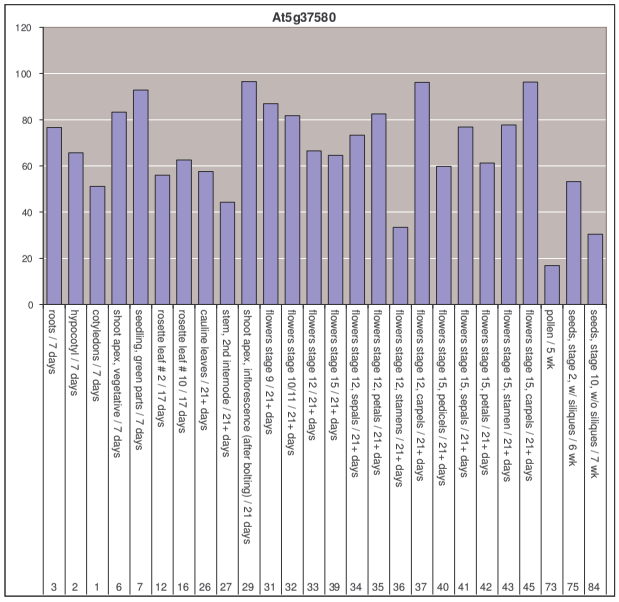
ankyrin repeat family protein



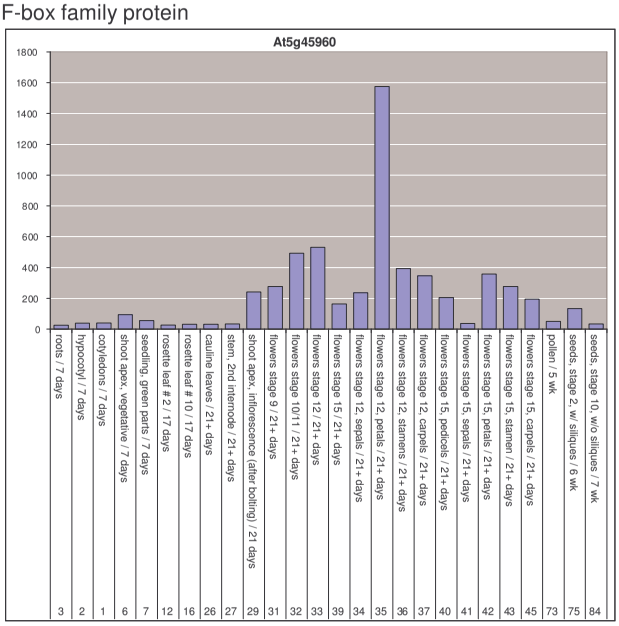
AAA-type ATPase family protein



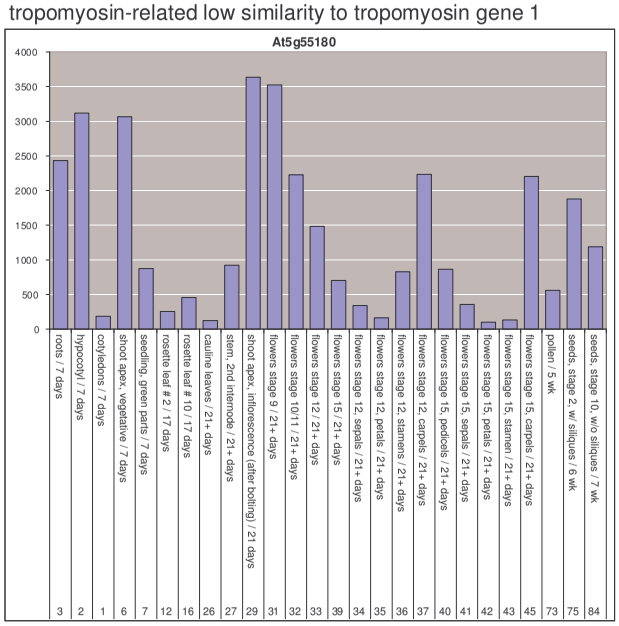
F-box family protein



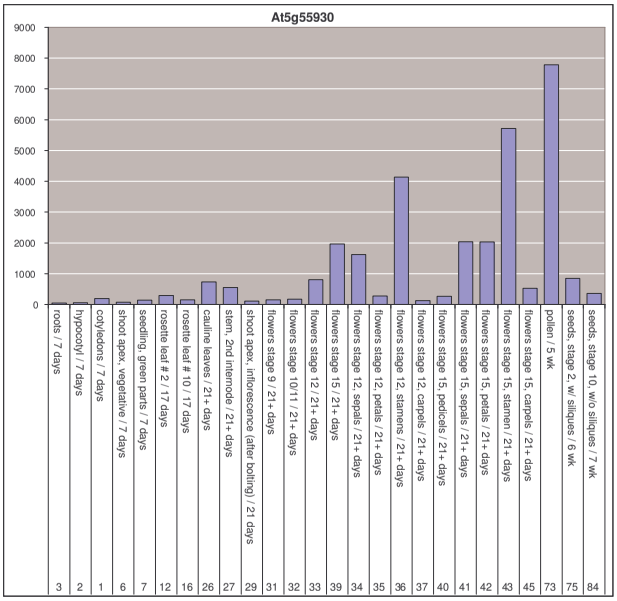
tropomyosin-related low similarity to tropomyosin gene 1



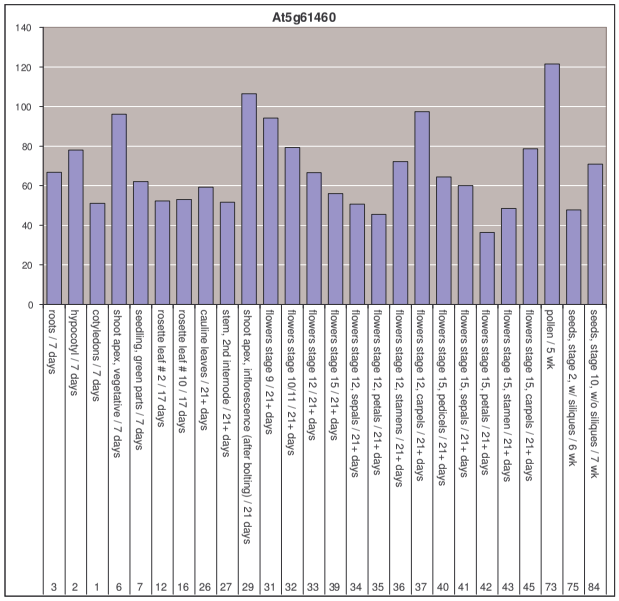
GDSL-motif lipase/hydrolase family protein



glycosyl hydrolase family 17 protein similar to elicitor inducible chitinase



oligopeptide transporter OPT family protein similar to SP|P40900 Sexual differentiation process protein isp4 {Schizosaccharomyces pombe}



structural maintenance of chromosomes (SMC) family protein very strong similarity to SMC-like protein (MIM)

8.4 Appendix D: Transgenic plants used in this study

Seed stock number	Gene	Line	Remarks
60001	*SPL2*	INRA 245C09	homozygous *spl2* knock-out, WS background
60002	*SPL2*	INRA 245C09	homozygous *spl2* knock-out, WS background
60003	*SPL2*	INRA 245C09	homozygous *spl2* knock-out, WS background
60005	*SPL2*	SALK 022235	homozygous *spl2* knock-out, Col background
60006	*SPL2*	SALK 022235	homozygous *spl2* knock-out, Col background
60007	*SPL2*	SALK 022235	homozygous *spl2* knock-out, Col background
60046	*SPL12*	SALK 017778	homozygous *spl12* knock-out, Col background
60047	*SPL12*	SALK 017778	homozygous *spl12* knock-out, Col background
60048	*SPL12*	SALK 017778	homozygous *spl12* knock-out, Col background
60066	*SPL15*	SALK 074426	homozygous *spl15* knock-out, Col background
60067	*SPL15*	SALK 074426	homozygous *spl15* knock-out, Col background
60068	*SPL15*	SALK 074426	homozygous *spl15* knock-out, Col background
60069	*SPL1*	SALK 070086	homozygous *spl1* knock-out, Col background
60070	*SPL1*	SALK 070086	homozygous *spl1* knock-out, Col background
60071	*SPL1*	SALK 070086	homozygous *spl1* knock-out, Col background
60075	*SPL11*	INRA 422H07	homozygous *spl11* knock-out, Col background
60076	*SPL11*	INRA 422H07	homozygous *spl11* knock-out, Col background
60077	*SPL11*	INRA 422H07	homozygous *spl11* knock-out, Col background
60087	*SPL15*	SALK 138712	homozygous *spl15* knock-out, Col background
60088	*SPL15*	SALK 138712	homozygous *spl15* knock-out, Col background
60089	*SPL15*	SALK 138712	homozygous *spl15* knock-out, Col

			background
	SPL9	5ABA33-H1	homozygous *spl9* knock-out, Col
60091			background
	SPL9	5ABA33-H1	homozygous *spl9* knock-out, Col
60092			background
	SPL9	5ABA33-H1	homozygous *spl9* knock-out, Col
60093			background
	SPL8	StK002-2-1	*35S::AlcR*; *AlcA::VP16:SPL8* double
60103			transgenic plants, Col background
	SPL8	StK002-2-4	*35S::AlcR*; *AlcA::VP16:SPL8* double
60104			transgenic plants, Col background
	SPL8	StK002-3-1	*35S::AlcR*; *AlcA::VP16:SPL8* double
60105			transgenic plants, Col background
	SPL8	StK002-3-3	*35S::AlcR*; *AlcA::VP16:SPL8* double
60106			transgenic plants, Col background
	SPL8	StK002-9-3	*35S::AlcR*; *AlcA::VP16:SPL8* double
60107			transgenic plants, Col background
	SPL8	StK002-9-4	*35S::AlcR*; *AlcA::VP16:SPL8* double
60108			transgenic plants, Col background
	SPL9	5ABA33-H1	homozygous *spl9 spl15* double knock-out,
60109	*SPL15*	SALK 074426	Col background
	SPL9	5ABA33-H1	homozygous *spl9 spl15* double knock-out,
60110	*SPL15*	SALK 074426	Col background
60112	*SPL1*	SALK 070086	homozygous *spl1 spl12* double knock-out,
	SPL12	SALK 017778	Col background
	SPL9	SALK N506573	homozygous *spl9* knock-out, Col
60117			background
	SPL9	SALK N506573	homozygous *spl9* knock-out, Col
60118			background
	SPL9	SALK N506573	homozygous *spl9* knock-out, Col
60119			background
	SPL2	SALK 022235	
	SPL9	5ABA33-H1	homozygous *spl2 spl9 spl15* tripple knock-
60120	*SPL15*	SALK 074426	out, Col background
	SPL2	SALK 022235	homozygous *spl2 spl9 spl15* tripple knock-
	SPL9	5ABA33-H1	out, Col background
60121	*SPL15*	SALK 074426	
	SPL2	SALK 022235	homozygous *spl2 spl9 spl15* tripple knock-
	SPL9	5ABA33-H1	out, Col background
60122	*SPL15*	SALK 074426	

8.5 Appendix E: Primer used in this study

Primer	Sequence (5'-3')	Remark
SPL8 target genes		
s111	GAGAGATTGATTCCGCCGAACC	At1g08210
s112	CCTTGTCCTAACCCAAAGATGCC	
S113	TAGAAGAGTCAACACCGTCCCC	At1g16510
S114	GAGCAACGAAGCCACAAGATTC	
S115	TCAACTGCTGCCTCAAAACCTG	At1g17460
S116	TCCTGGTGACAACAACTCTCCC	
S117	GCTTGTCTCGTCATCAAATCCG	At1g22030
S118	TCCTTATCCCCATCCTCCCAAC	
S119	GGGAGTGAGAATGAGAAGTTGGG	At1g44830
S120	AGTGATGTTGTCAAATACGGCG	
S121	GACGATGAAACAACTAATCCGCC	At1g56150
S122	CCATACTCTTGAGCCGACTGCTTC	
S123	CCACAACGATTTTCTCTCTCTCACG	At1g69760
S124	CAAAGCCCAACACTAAACCCATC	
S125	TCCCCACCACCATCAGAAACAG	At2g40330
S126	TCTTCCTTATCGTTACCCGCC	
S127	CGTGGACCAATGTTCGCAAC	At3g10570
S128	AAGACGACGCCGTTTTATCAGTC	
S129	AAGCCCATCCTTCAACATTGC	At3g15270
S130	TCCTCCTCTCATTGTGTCCAGC	
S131	ACATTTCGGTGTGGTCCCTGTG	At3g26200
S132	TTCCATCAGGCAAACTCCAATC	
S133	CCGCCTTCTCACTTATGCTTACAG	At3g45060
S134	CCATCAACGATAGTCCTTCCGC	
S135	CAACAACATAAAGAGTCACCAGGC	At3g50770
S136	GATTCCCCTAACTTCACAAGCATC	
S137	CACTGATGATACAAACCGACACG	At3g53950
S138	GAATCTCCGCTGTGGTGAAATC	
S139	GTAGGAGTCACCACTTTTGGCG	At3g57520
S140	TTCATCAACACCCTCGGCAG	
S141	CTAAGCGGTGGAAGTGAGAACG	At3g57780
S142	CGTGGGAATCTGATGCTCTGAC	
S143	CTCGGGTTCCAAAGTTGTTGTC	At3g63240
S144	GAGGAGAAGTGTGAAGCCAATCG	
S145	GGCTTGAAGAGCATCCAGAAGTC	At4g08040
S146	CCAACAGAACAAACCTGCGTTAC	
S147	ATTCTGATGGCTGAAACGAGC	At4g13100
S148	ATCTCTTCCCTCCACTTCCACCTC	
S149	AACTTGGCGTGGAAAGCAATAG	At4g19380
S150	CAACAGATGCGGCGTTCTAAG	

S151	CTGGTGGTGCTCGGTCAATAAC	At5g09440
S152	AAACAAACACATCCCTTTCCCC	
S153	CTAACACTGGAGAAGAGTTTCGGG	At5g14230
S154	CACAGTGTAAAGCATAAAACCCGC	
S155	CCTTCACCTACTTCGGTTTTCACAG	At5g17760
S156	ATTCAGCAGCCCCGATAACG	
S157	AATCAGCAAAGACTTCCTCCGAG	At5g27920
S158	CAAGCCAGACAAACTCACACGG	
S159	CGAAGGGATGGAACTCAATGG	At5g37580
S160	GCAGCAGTATGACTAAAGCCGC	
S161	CCTGTTCTTCATCTCCTTCTCATCC	At5g45960
S162	CTTGCTCCTTCTTTCCACAAACC	
S163	GAATCAAAGTCGTTGTCTCTCTCCC	At5g55180
S164	TGAATCGGACGGCAATCAGC	
S165	TCGTGTTAGAAGGCTTGAACGG	At5g61460
S166	AGGAGGAAGAGTAGTCTGAACTGGC	
Primer flanking T-DNA insertion sites		
st31	CCTTCATGGATACATCAGTCTAGTCCG	SALK 058642
st30	GCAACCCCGGATTGACGATAAATAC	
st33	CCCCTCTTTGACAATACTCGTC	SALK 070086
st32	CCATAGCTTGCTTAGCAGAATCAGTTCC	
st23	GATTACTCTCTTGAAGATACTCTCC	INRA 337H05
st24	GAGATGGAATCATAACAAATTACG	
st27	CGGTTCTTCATATGTTTGATAGT	INRA 245C09
st28	CATCAAACTCAGAGAGACAGTGGAACC	
st48	GCATGAATAAGCAGAGGGTTTTAATATAGG	SALK 022235
st49	CCAAAAGTCTTCAAAGTACGTTCGTTTAC	
st25	AGCTCCTCATGTTCGGATCTCTGGTC	INRA 173C12
st26	GCATTCCCTGCTAATGCAGCAGCA	
st14	CTTCTCTGTTCCTCTAATCGG	GABI-Kat 515E04
st15	CAGCACTGAATCCATTGG	
st65	GTCTTCTCTGTCGCAATCGCCACC	SALK 109908
st66	CCAGGAGGATGCATAGGAGGTTAAC	
st34	GCTGGAGGGCTATATCCGCCCAGG	SALK 093849
st35	CCAAGACAAATAAGTATTAGTTCAC	
st46	GGTGAAGGAATTGAATCTATAGTTCC	INRA 295G12
st47	GCTACACTGTTGGCAGAAACGACG	
st102	GCCACTTAGTTTTTGTGTGATGG	INRA 422H07
GC540	GAGGTTGACAGAAGAGAGAGAGCACGGTG	
st53	GGCACTGTTGATCCATCTCCTGATGCTGCG	SALK 017778
st52	GGTATAGGGAAGTTTTACTAGCTTGTTCC	
st98	GGACAAATCTTGATATTGCTTTGATTGC	SALK 079029
st99	GGGACAAAGAAAGTGGTGGTTGAAGC	
st16	GTTTTGCCAACAGTGTAGCAGG	GABI-Kat 567F10
st17	TAGAAGATGCTACTGTCGGTCG	

st82	GGATGAGGTAGGAGCTCAAGTGGC	GABI-Kat 566H06
st83	CCGCATTCTTACCTGCAGATCACAC	
st37	CGTAGCTGTCGTGGACTAGTGTCAATC	SALK 074426 & SALK 038712
st76	CGCCCGCGATGACTTCCCAAGTTTGGC	
st20	CTCTAATGCTCAGGTATGCTGCTAC	GABI-Kat 085F04
st38	CATCTGCCAATCATCCTTGGG	
st39	CCAATTATCCTCAATGTTTGTTGATATACG	SALK 020264
st21	TCACCGGCTACAACAGCCAACGGCG	
Left border primer for T-DNA's		
st29	CGGCTATTGGTAATAGGACACTGG	pGKB5 (FST)
SH72	ATATTGACCATCATACTCATTGC	pAC161 (GABI Kat)
ZY36	GCGTGGACCGCTTGCTGCAACT	pROK2 (SALK)
RT-PCR Primer to test knock-out lines		
GC200	GACTCAGTAGCAGCTTCTTCC	SPL1
GC201	CACACAGACGCAAACCGCAGC	
GC129	GCTGGTAGTTCAGGTTCTGA	SPL2
GC256	CTTGATGGTACGTGCTTCGAACTTGGC	
GC705	GGTCGGGTCAGTCGGGTCAGATACC	SPL9
GC638	ACTGGCCGCCTCATCACTCTTGTATCC	
GC540	GAGGTTGACAGAAGAGAGAGAGCACGGTG	SPL11
GC538	ATCTGCTAAGGGTTATCATCGTAAGCAC	
st52	GGTATAGGGAAGTTTTACTAGCTTGTTCC	SPL12
st53	GGCACTGTTGATCCATCTCCTGATGCTGCG	
GC665	AGCCATTGTAACCTTATCGGAGAATGAG	SPL15
GC712	AGAAGCAAGAACCGGGTCAATACC	
Primer used for StK002 (EtOH inducible *SPL8*)		
st56	AGGTCCCGGGATGACGAAAAACAATTACG	VP16-domaine, forward
st11	CTAATTACCTCGAGTCCTCCGGACC	VP16-domaine, reverse
st03		SPL8, forward
st55	GTAAAGCTTACCCGGGTCCGCTGGAG	SPL8, reverse
Primer used for StK005 (*pSPL9* construct)		
S255	ATATCCCGGGATTCTTGTCTCTTAATCTGTTTTGG	forward
S259	ATATGAGCTCGTTGTTGTTCTTCAGGAGACGAGTC	reverse
Primer used for StK004 (*pSPL15::GUS:SPL15* reporter construct)		
S109	GGAATTCGCTCTTCCCTCCTAGTTTCCATGGCG	*SPL15* forward
S110	GGAATTCCCAAACGGAAAGGCATTATCGGGC	*SPL15* reverse

S181	AAAAAGTACTATGGTACGTCCTGTAGAAACCCC	*GUS*, forward
S182	AAAAAGTACTTCATTGTTTGCCTCCCTGCTGCGG	*GUS*, reverse

9 Abbreviations

BD	Binding Domain
cDNA	Complementary Deoxyribonucleic Acid
Col-0	Columbia-0
DBD	DNA-Binding Domain
DNA	Deoxyribonucleic Acid
DNase	Deoxyribonuclease
dNTP	Deoxynucleoside Triphosphate
EtOH	Ethanol
g	Gram
GUS	β-Glucoronidase
IAA	Indole-3-Acetic Acid
kb	Kilo Base Pair
L	Liter
LD	Long Day
LRP	Lateral Root Primordium
min	Minute
mm	Millimeter
mRNA	Messenger Ribonucleic Acid
NPA	Naphtylphthalamic Acid
PCR	Polymerase Chain Reaction
RAM	Root Apical Meristem
RNA	Ribonucleic Acid
RPM	Rounds per Minute
RT	Room Temperature
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SAM	Shoot Apical Meristem
SBP	Squamosa Promoter Binding Protein
SD	Short Day

Sec	Second
SPL	Squamosa Promoter Binding Protein Like
WT	Wild Type
YFP	Yellow Fluorescent Protein
β-ME	β-Mercaptoethanol
µl	Mycroliter
M	Molar
QC	Quiescent Centre

10 Nomenclature

The genotype is written in italics.

The wild-type genotype is written in capitals (e.g. *SPL15*).

The mutant genotype is written in lower case letters (e.g. *spl15*).

The polypeptide products of genes are written in non-italics, capital letters (e.g. SPL15).

11 Eidesstattliche Erklärung

Köln, 2006

„Ich versichere, dass ich die von mir vorgelegte Dissertation selbständig angefertigt, die benutzten Quellen und Hilfsmittel vollständig angegeben und die Stellen der Arbeit – einschließlich Tabellen, Karten und Abbildungen –, die anderen Werken im Wortlaut oder dem Sinn nach entnommen sind, in jedem Einzelfall als Entlehnung kenntlich gemacht habe; dass diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; dass sie – abgesehen von den unten angegebenen Teilpublikationen – noch nicht veröffentlicht worden ist sowie, dass ich eine solche Veröffentlichung vor Abschluß des Promotionsverfahrens nicht vornehmen werde. Die Bestimmungen der Promotionsordung sind mir bekannt. Die mir vorgelegte Dissertation ist von Herrn Prof. Dr. Heinz Saedler betreut worden."

Stefan Schwarz

12 Lebenslauf

Zur Person

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