The SBP-Box Gene *SPL8* Affects Reproductive Development and Gibberellin Response in Arabidopsis

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Yan Zhang

aus Yining, China

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Berichterstatter:	Prof. Dr. Heinz Saedler
	Prof. Dr. Ulf-Ingo Flügge
Prüfungsvorsitzender:	Prof. Dr. Wolfgang Werr
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Abbreviations

Ab	Antibody
AD	Activation domain
BD	binding domain
□-ME	□-mercaptoethanol
BR	Brassinosteriod
cDNA	Complementary deoxyribonucleic acid
Col-0	Columbia-0
dCTP	Deoxycytidine triphosphate
DAG	Days after germination
DBD	DNA-binding domain
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxynucleoside triphosphate
EDTA	Ethylenediamine tetraacetic acid
EtOH	Ethanol
FDA	Fluorescein diacetate
g	gram
GA	Gibberellic acids/gibberellin
GFP	Green fluorescent protein
GM	Germination media
hr	hour
JA	Jasmonic acid
kb	Kilo base pair
L	Liter

LD	Long day
min	Minute
mm	Milimeter
MPIZ	Max-Planck-Institute für Züchtungsforschung
mRNA	Messenger ribonucleic acid
NLS	Nuclear localization signal
OD_{600}	Optical density at 600 nm
PAC	Paclobutrazol
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PIPES	piperazine-1,4-bis2-ethanesulfonic acid
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Rounds per minute
RT	Room temperature
RT-PCR	Reverse transcription/polymerase chain reaction
SBP	Squamosa promoter binding protein
SD	Short day
SDS	Sodium dodecyl sulfate
sec	Second
SEM	Scanning electron micrograph
SPL	Squamosa promoter binding protein like
WT	wild-type
Y2H	Yeast two hybrid

Nomenclature

In this study, genotypes are written in italics;

The wild-type genotype is in capitals (e.g., *SPL8*);

The mutant genotype is in lowercase letters (e.g., *spl8-1*);

The polypeptide products of genes are written in non-italic capitals (e.g., SPL8).

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1. General Introduction

Plants, the angiosperms in particular, form major sources of food, fuel, drugs, fibers, building material and so on, and are essential for human life. Angiosperms or flowering plants, propagate through the recognition and interaction of male and female reproductive organs, respectively the stamens and carpels. Therefore, a full understanding of the reproductive development is essential for plant breeding and agriculture. Compared to the carpel, development of the stamen attracted more interests due to the fact that male sterility can be used to facilitate hybrid production via cross-pollination, which has a significant impact on agriculture.

Because development requires precise and dynamic regulatory networks, it is important to understand the role of transcription factors as they are major determinants in regulating temporal and spatial gene expression. Transcription factors are proteins that show sequence-specific binding to DNA and are capable of activating/repressing transcription. Transcription factors are grouped into families according to their conserved DNA-binding domain (DBD). In the model plant Arabidopsis, over 5% of the genome is dedicated to code for more than 1500 transcription factors classified into over 30 families (Riechmann et al., 2000). Around half of these transcription factor families are plant-specific, suggesting that their members play roles in plant-specific processes.

1.1 SBP-box genes encode putative transcription factors

A highly interesting family of plant-specific transcription factors is the SBP-box family, designated according to the highly conserved SBP-domain (Squamosa promoter Binding Protein). SBP-box genes were first identified in *Antirrhinum majus* for the capacity of their protein products to bind to the promoter region of the floral meristem identity gene *SQUAMOSA* (Klein et al.,

1996; Huijser et al., 1992). Since then, SBP-box genes have been found in diverse plant species (Cardon et al., 1999; Lännenpää et al., 2004) and 16 to be present in the model species *Arabidopsis thaliana* where they are known as *SPL1* to *SPL16* (for SBP-domain Protein Like; Cardon et al., 1999).

SBP-box genes can be grouped into distinct subfamilies according to their genomic structures and sequence features. One subfamily contains genes with relatively large genomic loci organized in 10 or more exons. Their protein products share extensive sequence similarity downstream of the conserved DNA-binding domain where ankyrin repeat-like sequences, probably involved in protein-protein interaction, can be found. Arabidopsis *SPL1*, *7*, *12*, *14* and *16* belong to this subfamily (Fig. 1-1). These genes are constitutively expressed in Arabidopsis according to the microarray database Genevestigator (Zimmermann et al., 2004), suggesting that their activities are likely regulated post-transcriptionally rather than at the level of transcription.

Members of the second subfamily are relatively small with less than 5 exons, including Arabidopsis *SPL2*, *3*, *4*, *5*, *6*, *9*, *10*, *11*, *13*, and *15* (Fig. 1-1). Their protein products are rather heterogeous outside the SBP-domain. Most interestingly, transcriptional activity of these *SPLs* genes correlates with the floral transition and has been shown to become upregulated during floral induction, strongly suggesting that they are involved in vegetative to reproductive growth transition (Cardon et al., 1997; 1999; Schmid et al., 2003).

Recently, members of this second subfamily regained interest as they were predicted to be targets of miRNA156 and miRNA157 (Rhoades et al., 2002). MiRNAs are 21-24 nucleotide small RNAs generated by precise processing of primary transcripts of non-protein-coding genes (Bartel, 2004). Two mechanisms are utilized by miRNAs to regulate the expression of their target genes. MiRNAs either inhibit the translation or result in cleavage followed by degradation of their target mRNAs (Bartel, 2004; Dugas and Bartel, 2004). Genes targeted by miRNAs are involved in diverse developmental aspects, including but not limited to, hormone response,

patterning, control of cell division, flowering, and environment/stress responses, indicating that miRNAs play important roles in plant development (for review, see Kidner and Martienssen, 2005). Constitutive expression of miRNA156 reduced the transcript levels of *SPL* genes harbouring the corresponding miRNA target site, suggesting that the miRNA recognition motif within these *SPL*s is functional (Schwab et al., 2005).

Another subfamily, in Arabidopsis represented by *SPL8* (Fig. 1-1), contains SBP-box genes with genomic structures very much like the second subfamily members except that they do not contain the miRNA recognition motif.

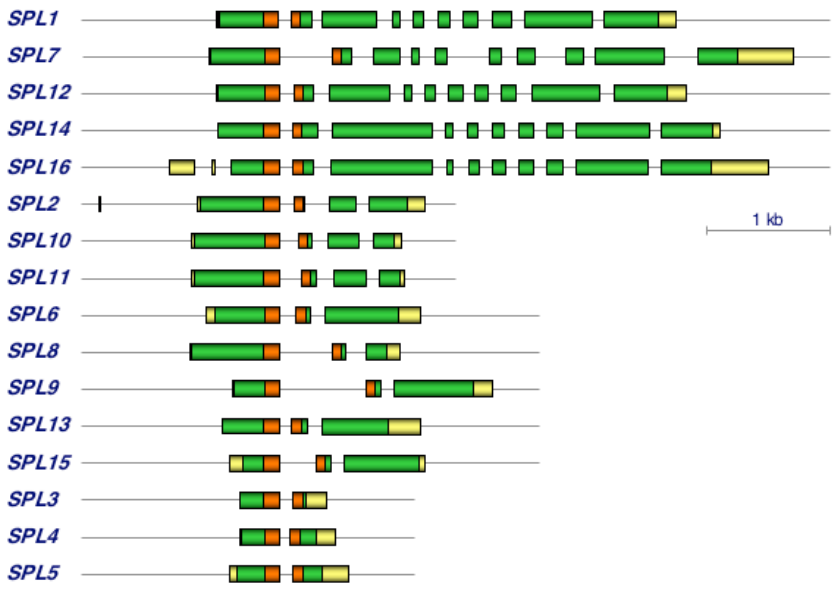

Figure 1-1. Schematic illustration of genomic structures of the SBP-box genes in Arabidopsis. Open boxes represent exons; lines, introns; red bars, SBP-box; Yellow bars, ankyrin repeats.

SBP-domain proteins are believed to be transcription factors due to their characteristic and novel zinc-dependent DBD, SBP-domain (Klein et al., 1996; Yamasaki et al., 2004). The SBP-domain spans 76 amino acid residues

and contains two zinc-binding sites with eight Cys or His residues in a Cys3HisCys2-HisCys or Cys6HisCys sequence motif. The first four residues coordinate one and the last four a second zinc ion (Yamasaki et al., 2004; Fig. 1-2). The SBP-domain binds specifically to sequences containing a GTAC core (Birkenbihl et al., 2005).

In addition to their sequence-specific DBD, SBP-domain proteins also share a highly conserved bipartite nuclear localization signal (NLS), another feature characteristic for transcription factors. The NLS is located at the C-terminus of the DBD (Fig. 1-2) and confirmed to be functional in transient expression assays with fluorescent protein fusion constructs (Birkenbihl et al., 2005). Within the NLS, a highly conserved serine has been predicted to be a putative phosphorylation site (Fig. 1-2), suggesting that subcellular localization of SBP-domain proteins may be under the control of post-transcriptional modifications, such as phosphorylation (Birkenbihl et al., 2005).

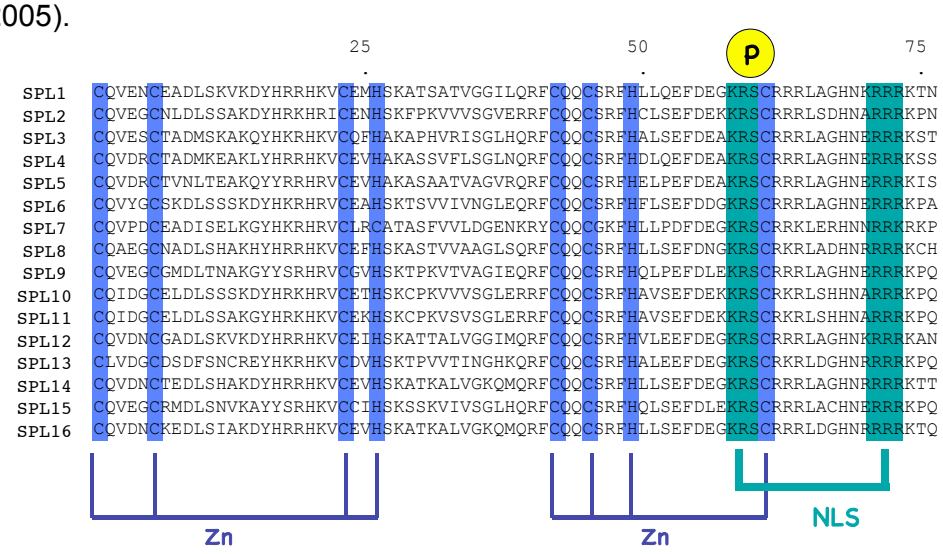

Figure 1-2. Sequence alignment of SBP-domains of SPLs, showing the two conserved zinc fingers, NLS, and the conserved serine, which can be phosphorylated. Picture modified from Yamasaki et al., 2004 and Birkenbihl et al., 2005.

1.2 SBP-box genes may play diverse roles in plant development

Although it has been almost ten years since the identification of the SBP-box gene family, not much has been uncovered about their roles in plant development. Till now, the function of only five SBP-box genes has been described through analysis of either their loss-of-function or gain-of-function mutants. They include the maize genes *LIGULELESS1* (*LG1*) and *TEOSINTE GLUME ARCHITECTURE* (*TGA1*), and Arabidopsis *SPL3, 8* and 14.

LIGULELESS1 (*LG1*) is the first SBP-box gene for which a loss-of-function mutant phenotype was described. Maize *lg1* is defective in the differentiation of ligules and auricles during maize leaf organogenesis (Becraft et al., 1990). The maize ligule and auricle are structures that develop at the boundary of the leaf sheath and blade. In the absence of *LG1* gene expression, ligule and auricle are not formed, and a sharp boundary does not develop between sheath and blade (Becraft et al., 1990). With the help of an *Activator* (*Ac*) transposable element as molecular tag, Moreno and coworkers (1997) isolated and cloned a novel *lg1* allele, *lg1-m1*. Analysis of somatic revertant sectors confirmed that *LG1* acts in a cell-autonomous fashion. cDNA cloning as well as RT-PCR analysis indicated that *LG1* is expressed at very low levels in the ligular region of developing maize leaf primordia, perhaps as early as plastochron 6 or earlier. Cellular localization studies in a heterologous system demonstrated that the LG1 is localized exclusively in the nucleus as expected for a transcription factor (Moreno et al., 1997).

The second SBP-box gene in maize has recently been characterized from an attempt to find out the molecular basis of morphological difference between maize and teosinte kernel (Wang et al., 2005). Several nucleotide changes in the promoter and coding sequences of *TEOSINTE GLUME ARCHITECTURE* (*TGA1*) were proposed to be responsible for the evolution of maize inflorescence architecture (Wang et al., 2005).

Arabidopsis *SPL3* belongs to the second SBP-box subfamily, with short genomic sequences and a miRNA156 recognition motif. *SPL3*, together

with its close homolog *SPL4* and *SPL5*, is slightly different from the other members of this subfamily for containing the miRNA156/157 recognition site in its 3' UTR rather than in the coding region (Rhoades et al., 2002). *SPL3* is highly expressed in vegetative and inflorescence apex, floral meristem, leaf and floral primordia (Cardon et al., 1997). A role for *SPL3* in floral induction was uncovered in transgenic lines where constitutive expression of an *SPL3* transgene in the absence of a functional miRNA156/157 recognition motif caused early flowering both under LD and SD conditions (Cardon et al., 1997). Furthermore, *SPL3* as well as *SPL4* and *SPL5* become dramatically upregulated in response to long day floral induction (Schmid et al., 2003), again strongly suggesting a role in the vegetative to reproductive phase transition.

The regulation of *SPL3* by miRNA156 has recently been proved (Schwab et al., 2005; Park et al., 2005). Constitutively expressing miRNA156 reduced *SPL3* transcript levels in the shoot apex at the floral transition (Schwab et al., 2005). On the other hand, *SPL3* transcript levels dramatically increased in *hasty* (*hst*) mutants, which are defective in the cytoplasmic accumulation of miRNA156 and other miRNAs (Park et al., 2005).

One of the largest SBP-box genes, *AtSPL14*, has recently been characterized as it was identified in a genetic screen for mutants conferring resistance to the programmed cell death (PCD)-inducing fungal toxin fumonisin B1 (FB1). The corresponding mutant, *fbr6*, displayed reduced *SPL14* transcript levels and besides conferring resistance to FB1, also showed defects in plant development, like elongated petioles, enhanced leaf margin serration, and hastened juvenile to adult phase transition (Stone et al., 2005). These observations suggest that *AtSPL14* is not only involved in PCD-related plant defense response, but also in normal developmental processes governing plant morphogenesis and phase transition.

SPL8 is the first Arabidopsis SBP-box gene for which a loss-of-function phenotype has been published (Unte et al., 2003). Three *spl8* allelic mutants were identified in an *Arabidopsis thaliana* population mutagenized by

a transgenically introduced *En* transposon from maize. As all three mutants displayed semi-sterility (Fig. 1-3A, 3B), an important role for *SPL8* in plant reproductive development has been suggested (Unte et al., 2003). The expression pattern of *SPL8* correlates with its loss-of-function phenotype such that *SPL8* is mainly detectable in inflorescences, and highly expressed in anthers at early developmental stages (Cardon et al., 1999; Unte et al., 2003).

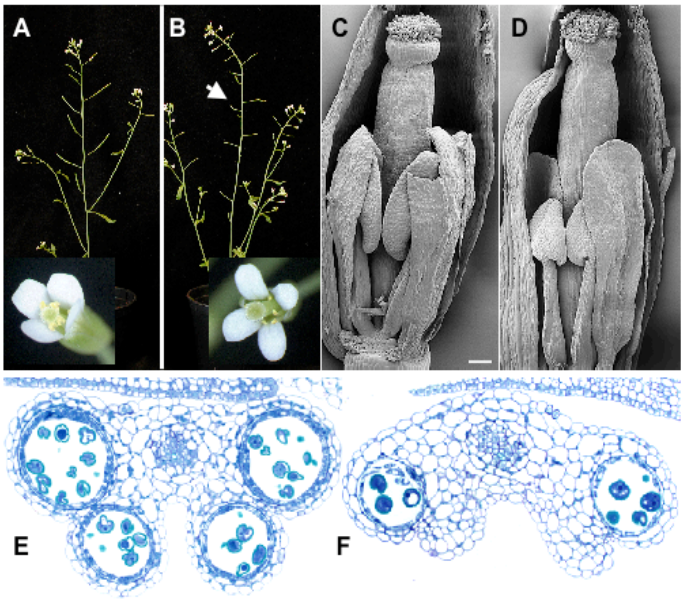

Figure 1-3. *SPL8* is involved in plant reproductive growth. Pictures adapted from Unte et al., 2003. (A), (C), (E), wild-type; (B), (D), (F), *spl8-1* mutant.
(A) and (B) plants in reproductive phase. White arrowhead indicates a sterile silique. Insets are close-up views of flowers.
(C) and (D) SEM images of floral buds with two sepals and one petal removed.
(E) and (F) Cross sections of anthers at stage 10-11.

Phenotypical alterations during vegetative growth have not been reported for *spl8* mutants (Unte et al., 2003). However, after the floral induction, the early arising flowers display complete sterility while later ones are only partially sterile (Fig. 1-3A, 3B). Flowers of *spl8* mutants have structurally abnormal stamens with short filaments and small anthers (Unte et al., 2003; Fig. 1-3C, 3D). Transverse sections of *spl8* and wild-type developing anthers revealed that *SPL8* loss-of-function affects anther

development at very early stages starting from the initiation of archesporial cells (Unte et al., 2003). The failure of *spl8* mutant to properly form archesporial cells in each corner of the anther results in anthers with less than four pollen sacs or even no pollen sacs formed at all (Fig. 1-3E, 3F). Megasporogenesis has also found to be disturbed, resulting in a reduced seed set even after pollination of *spl8* mutant flowers with ample wild-type pollen (Unte et al., 2003).

1.3 Formation of the male reproductive organ

Male reproductive development starts with the initiation and formation of stamens in the third whorl of the flower. According to the well-established ABC model (Theissen and Saedler, 2001), B and C gene functions are required for stamen identity. In Arabidopsis, genes fulfilling the B function are *APETALA3* (*AP3*) and *PISTILATA* (*PI*), while *AGAMOUS* (*AG*) represents the C function. Mutations in either of these homeotic genes cause defects in stamen identity (Bowman et al., 1989) and their ectopic expression may lead to ectopic stamen formation (Mizukami and Ma, 1992; Krizek and Meyerowitz, 1996).

After the establishment of stamen identity, the three cell layers of the stamen primordium start to undergo cell division and differentiation, which results in the formation of specialized anther sporocytic tissues. L1 cells continue to divide anticlinally and form the anther epidermis, while cell division and differentiation in L3 give rise to the connective and vascular tissues. Division of L2 cells results in the development of the internal cell layers of the anther lobes, i.e. the pollen sacs, the central cells of which will undergo meiosis and eventually develop into pollen (Goldberg et al., 1993). Anther development in Arabidopsis has been divided into 12 stages according to morphological and cellular features seen in transverse sections through developing anthers (Sanders et al., 1999). Before stage 6, cell division and differentiation produce a four-lobed structure with microsporocyte mother cells

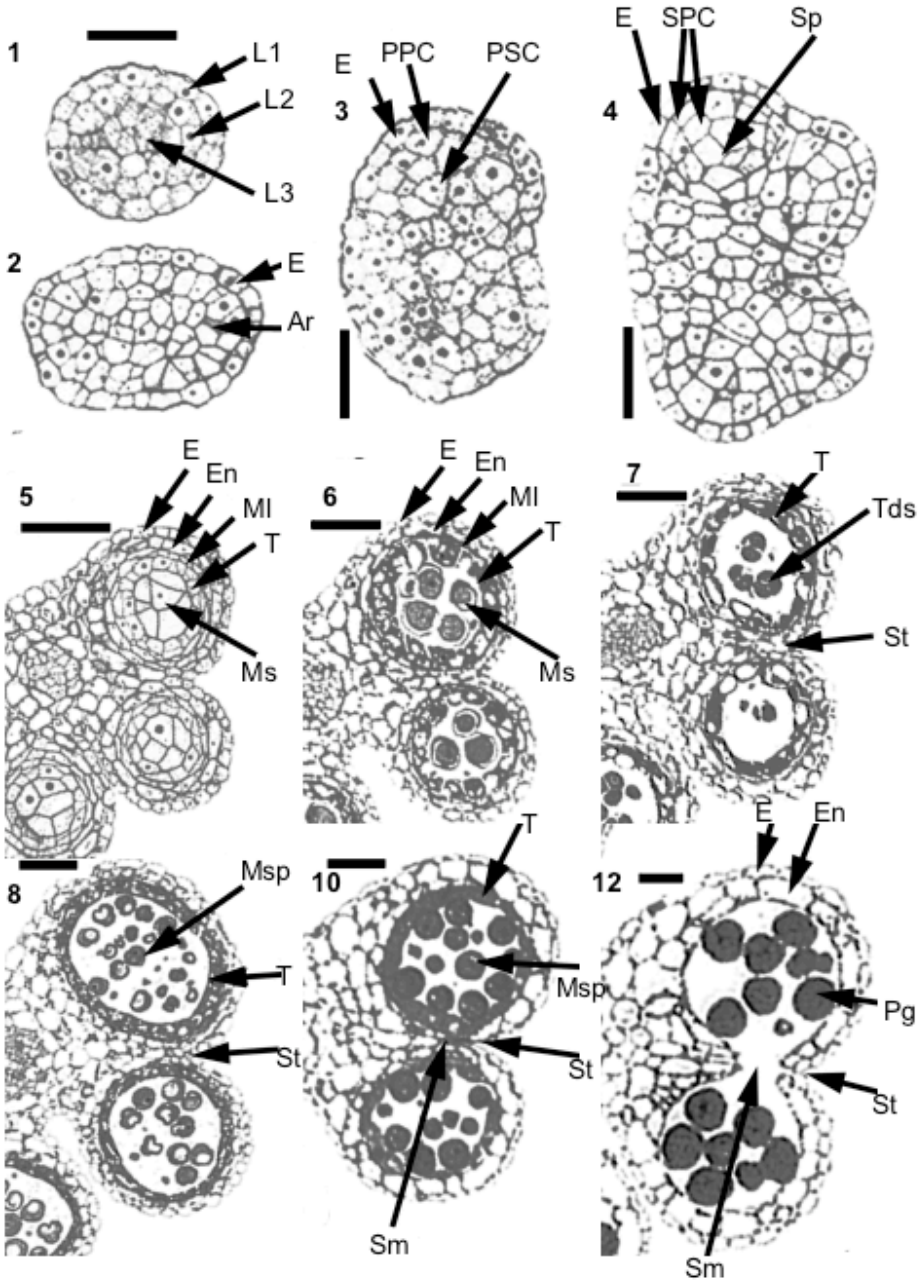

Figure 1-4. The anther developmental stages of Arabidopsis. Pictures modified from Ma, 2005. Ar, archesporial cell; E, epidermis; En, endothecium; L1, L2 and L3, the three cell layers in stamen primordia; Ml, middle layer; Ms, microsporocytes; Msp, microspores; Pg, pollen grain; PPC, primary parietal cell; PSC, primary sporogenous cell; Sm, septum; Sp, sporogenous cells; SPC, secondary parietal cell; St, stomium; T, tapetum; Tds, tetrads. Scale bar = 25 □m.

(MMCs) surrounded by tapetum, middle layer, endothecium and epidermis, from interior to exterior sequentially (Sanders et al., 1999). Starting from stage 6, MMCs undergo meiosis and stage 7 shows the completion of meiosis in the formation of tetrads, which release microspores at stage 8. Microspores develop into pollen during stage 9-10, in which nutrients are provided by a simultaneously degenerating tapetum. Pollen mitotic division gives rise to tricellular haploid pollen grains at anther stage 11-12. Stage 13 and 14 are the final stages during anther development, and happen when the female reproductive organ starts to become receptive. Anther dehiscence at these stages enables the release of mature pollen grains onto the stigmatic tissue of the carpel and anther cells start to go through programmed cell death (Reviewed by Ma, 2005).

1.4 Genes involved in Arabidopsis anther development

The modern microarray technique allows high throughput studies on global gene expression in specific tissues. According to some recent microarray studies, over thousands of genes are expressed specifically in anthers (Amagai et al., 2003; Zik and Irish, 2003). These genes mainly encode for proteins involved in transcriptional regulation, metabolism of protein, starch, and sucrose, cell wall biosynthesis and expansion, lipid translocation, and cytoskeleton structure. Due to their expression profiles, these anther-specific genes are likely to be involved in anther development and therefore worth to investigate following reverse genetic approaches.

Forward genetics has also been extensively used and several successful screens revealed many mutants with male sterility or semi-sterility phenotypes (Sanders et al., 1999; 2000; Sorensen et al., 2002). Cloning of genes responsible for such phenotypes revealed many genes important for diverse stages of Arabidopsis anther development.

Arabidopsis *SPOROCYTELESS* (*SPL*)/*NOZZLE* (*NZZ*) is involved in early sporogenesis. Mutations in *SPL*/*NZZ* do not affect the formation of archesporial cells but disrupt the following cell division. As a result,

sporogenous cells and tapetum are absent, leading to sterility (Schiefthaler et al., 1999; Yang et al., 1999). *SPL*/*NZZ* encodes a nuclear localized protein proposed to be a transcriptional regulator (Schiefthaler et al., 1999; Yang et al., 1999). A recent finding provides evidence that *SPL*/*NZZ* functions downstream of the C function gene *AGAMOUS* (*AG*) (Ito et al., 2004).

EXCESS MALE SPOROCYTES1 (*EMS1*)/*EXTRA SPOROGENOUS CELLS* (*EXS*) functions at a later stage than *SPL*/*NZZ*. The *ems1*/*exs* mutants are defective in the differentiation of anther cell layers, such that tapetal cells are absent and replaced by extra sporocytes (Canales et al., 2002; Zhao et al., 2002). *EMS1*/*EXS* encodes a putative leucine repeat receptor protein kinase (Zhao et al., 2002), proposed by Ma (2005) to function in a signaling pathway with *TAPETUM DETERMINANT1* (*TPD1*). The mutant *tpd1* displays an anther phenotype similar to that of *ems1/exs* (Yang et al., 2003). In addition, *TPD1* encodes a small protein with a putative secretion signal peptide (Yang et al., 2003). In addition to the similar expression patterns and mutant phenotypes, *EMS1/EXS* and *TPD1* have recently been shown to be in the same genetic pathway regulating tapetum identity and maintenance (Yang et al., 2005).

A few other genes were also found to be involved in cell division and differentiation processes within anthers. Anthers of *aborted microspores* (*ams)* mutants display degenerating tapetum and microspores immediately after meiosis (Sorensen et al., 2003). Microspores in the *male sterile1* (*ms1*) mutant started to degenerate during stage 8 when wild-type microspores are released from the tetrads (Wilson et al., 2001). A tapetum-specific MYB gene, *AtMYB103*, affects tapetum morphology and pollen development when mutated (Higginson et al., 2003).

Another group of mutants is disrupted in meiosis. Meiosis forms the basis for sexual reproduction and results in the formation of haploid cells developing into gametes. In the anthers of angiosperms, meiosis gives rise to haploid micro- and macrospores, the male and female gametophytes respectively (Ma, 2005). Arabidopsis meiotic mutants include *syn1*/*dif1*,

defective in chromosome condensation and pairing (Bai et al., 1999; Bhatt et al., 1999), and *swi1*/*dyad*, defective in sister chromatid cohesion (Mercier et al., 2001; Agashe et al., 2002).

Successful reproduction needs proper developmental timing of pollen release through anther dehiscence. This process starts with the deposition of fibrous bands in endothecial and connective cells of the anther, resulting in cell wall thickening and strengthening. Finally, through expansion of the endothecial layer and simultaneous degeneration of specialized tissues, i.e. the septum and stomium, the anther wall will break and release pollen grains (Sanders et al., 1999). Structural male sterility, therefore, is observed in mutants affecting these processes. For instance, the Arabidopsis mutant *ms35* fails to thicken the endothecial cell wall, leading to an anther non-dehiscence phenotype, although septum and stomium degenerated normally (Dawson et al., 1999). Recently, it was found that *ms35* represents a mutation in an R2R3-type MYB gene *AtMYB26* (Steiner-Lange et al., 2003).

Anther dehiscence was also considered a process of programmed cell death (PCD). A mutant of *PROMOTION OF CELL SURVIVAL 1* (*PCS1*), encoding an aspartic protease, has been identified in a reverse genetic screen and shows increased PCD, leading to aborted sporocytes (Ge et al., 2005). On the other hand, septum and stomium of *PCS1* gain-of-function anthers do not degenerate, resulting in a failure of anther dehiscence (Ge et al., 2005).

1.5 Phytohormones involved in anther development

Phytohormones, including gibberellic acids (GAs), brassinosteroids (BRs), jasmonic acids (JAs), ethylene, auxins and cytokinins, also play key roles in anther development.

GAs regulate anther development in varying ways depending on their concentrations and plant species. GA-deficiency blocks anther development after meiosis in plants as Arabidopsis and petunia (Izhaki et al., 2002; Cheng

et al., 2004). In others, GA-deficiency causes pre-meiotic anther developmental defects, such as in tomato (Jacobsen and Olszewski, 1991).

In Arabidopsis, GA-deficiency does not affect early stages of microsporogenesis and meiosis is completed successfully, leading to the formation of tetrads (Cheng et al., 2004). However, starting from stage 7, microspores in GA-deficient anther are not properly released and pollen sacs fail to expand. The tapetum remains at the vacuolar stage and degenerates subsequently together with the microspores and pollen sacs (Cheng et al., 2004). GA-deficiency also results in shortened stamen filaments due to a reduction in cell elongation (Cheng et al., 2004). GA-insensitive mutants, such as *35S::GAI* transgenic plants, have similar anther defects to those of GA-deficient mutants (Huang et al., 2003). Several MYB transcription factors have been proposed as positive regulators in GA-dependent anther development (Millar and Gubler, 2005).

On the other hand, sterility was also reported for GA overdose (Colombo and Favret, 1996) and for constitutive GA response caused either by removing *SLR1* function in rice (Ikeda et al., 2001), by removing the function of a negative GA signaling regulator SPINDLY in Arabidopsis (Jacoben and Olszewski, 1993), by overexpressing a positive GA signaling regulator OsGAMYB in barley (Murray et al., 2003), or by overexpressing a soluble GA receptor GID1 in rice (Ueguchi-Tanaka et al., 2005). Unlike GA deficiency/insensitivity, GA overdose/constitutive response cause anther non-dehiscence in both species studied so far (Colombo and Favret, 1996; Murray et al., 2003).

BRs are another group of phytohormones essential for anther development. BR-deficient mutants, such as *dwf4* and *dwarf1*, show a reduction in stamen filament elongation, leading to sterility (Azpiroz et al., 1998; Choe et al., 1999). When mutated, the BR-biosynthetic gene, *CPD*, causes the failure of pollen to germinate (Szekeres et al., 1996). Mutants defective in BR-signaling, brassinosteroid-insensitive mutants *bri1* and *det2*

for instance, display male sterility as well (Chory et al., 1991; Clouse et al., 1996).

JAs are mainly known for plant resistance to pathogen, but they also play major roles in plant reproductive growth. Mutants defective in JA-biosynthesis or JA-signaling typically display shortened stamen filaments, delayed anther dehiscence, and non-viable pollen (reviewed by Scott et al., 2004). Sterility of the former class, including Arabidopsis *delayed dehiscence1*, *defective in anther dehiscence1* (*dad1*), and *opr3*, can be recovered by exogenous application of JAs (Sanders et al., 2000; Stintzi and Browse, 2000; Ishiguro et al., 2001), whereas sterility caused by JA-insensitivity, such as that of *coi1* and *coronatine-insensitive1*, cannot be rescued by exogenous JAs (Feys et al., 1994; Xie et al., 1998).

Ethylene also affects anther dehiscence when its concentrations and tissue sensitivity are perturbed. Ethylene-insensitive tobacco plants generated by expression of the mutant *etr1-1* ethylene-receptor allele from *Arabidopsis thaliana* or by treatment with the ethylene-perception inhibitor 1-methylcyclopropene have structurally normal anthers. But anther dehiscence is delayed such that pollen release does not synchronize with flower opening (Rieu et al., 2003).

Although auxins have the most diverse effects on plant development, they are less known for their role in anther development than the other phytohormones. Only a few reports mentioned that auxins might regulate anther development as deduced from the male sterility of some auxin-insensitive mutants. For instance, flowers of *auxin-resistant1* (*axr1*) produce less pollen and the stamen filaments fail to elongate, resulting in reduced male fertility (Lincoln et al., 1990). In another case, a mutant defective in *AUXIN RESPONSE FACTOR2* (*ARF2*) has been reported to display a basal-petal decrease in male fertility (Okushima et al., 2005).

Cytokinins have been implicated in the regulation of anther development since disturbed cytokinin metabolism has been found to result in post-meiotic pollen abortion in Arabidopsis (Gaillard et al., 1998).

Overexpression of *CKX1*, a gene involved in oxidative cytokinin degradation, causes male sterility in maize (Huang et al., 2003). Cytokinin-deficient transgenic Arabidopsis plants, created by overexpressing genes responsible for cytokinin catabolism, display a basal-petal decrease in male fertility (Werner et al., 2003). These observations suggest that cytokinin concentration has to be maintained to facilitate anther development.

Although individual phytohormones regulate anther development in different ways, anther development actually relies on the relative levels of these phytohormones (Sawhney and Shukla, 1994). In some cases, anther developmental defects caused by the deficiency of one hormone can be reversed by the application of another (Huang et al., 2003). In other cases, different hormones share the same regulatory components whose mutation conferred male sterility, like in the case of the *axr1* mutant, which is insensitive to both auxin and JAs (Tiryaki and Staswick, 2002). Phytohormones can also affect each other by regulating the expression of hormone biosynthetic genes. For instance, gibberellin A_1 biosynthesis is promoted by auxin in pea by upregulating the expression of the GA biosynthetic gene *PsGA3ox1* (Ross et al., 2000). In addition, the action of several hormones may converge on the same set of downstream components, such as the DELLA proteins acting as points of convergence for auxins, ethylene and GAs (Archard et al., 2003; Fu et al., 2003).

1.6 Project objective: functional analysis of *SPL8*

The project described in this thesis aimed at a better understanding of *SPL8* functionality and its involvement in the molecular/genetic mechanisms regulating anther development.

SPL8 loss-of-function mutants are defective in anther development, stamen elongation and trichome density, three developmental aspects regulated by GAs in Arabidopsis (Cheng et al., 2004; Perazza et al., 1998). Interestingly, *SPL8* transcript level was upregulated up to 5 fold in the apex of GA-deficient mutant *ga1-3* after the addition of bioactive GAs (M. Schmid and D. Weigel, personal communication). These observations hinted the

possibility that the regulation of *SPL8* on anther development is through phytohormone GAs. To test the possibility, *SPL8* gain-of-function should first be generated. A molecular and genetic analysis of *SPL8* gain-of-function in comparison to *SPL8* loss-of-function would be useful in addressing this question.

As described above, the subcellular localization of SBP-domain proteins may be regulated by post-transcriptional modificationmediated through an evolutionarily conserved serine residue in the vicinity of the NLS. SPL8 also contains the highly conserved serine residue in its SBP-domain. Accordingly, the second part of this project was to explore the effect of a potential post-transcriptional modification on the subcellular localization of SPL8.

The transcriptional regulation of transcription factors on their target genes usually requires the presence of other protein factors. In some cases, transcription factors execute their regulatory function in the form of protein complexes. Furthermore, the identification of protein partners would allow a protein of interest to be put in a regulatory network, facilitating the understanding of its functionality. The third part of the project hence aimed at the identification of proteins capable of interacting with SPL8.

Overall, functional analysis of SPL8 through a combination of molecular, cellular and genetic techniques would contribute to a better understanding of its role in plant development and anther development.

2. Material and Methods

2.1 Material

2.1.1 Antibiotics

Antibiotics	Stock Conc. (mg/ml)	Stock Solvent	Final Conc. (μg/ml)	
			E.coli	Agrobacteria
Gentamycin	10	H_2O	10	40
Rifamycin	50	methanol	100	100
Spectinomycin	50	H_2O	50	100
Kanamycin	12.5	H_2O	25	25

Table 2-1: Antibiotics used in this study.

2.1.2 Antibody

The optimal dilution for the primary SPL8 antibody SPL8-Ab was 1: 3000. The second antibody was horseradish peroxidase-labelled anti-rabbit antibody with the optimal dilution 1: 10000.

2.1.3 Bacteria strains

E. coli strains:

DH5α (Invitrogen, USA)

Genotype: *sup*E44 Δ*lac*U169(φ80*lac*ZΔM15) *hsd*R17 *rec*A1 *end*A1 *gyr*A96 *thi*-1 *rel*A1

TOP10 (Invitrogen, USA)

Genotype: F^- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L(Str^R) *end*A1 *nup*G

Agrobacterium tumefaciens strain:

GV3101 (pMP90), Rifamycin and Gentamycin resistance

2.1.4 Yeast strains

AH109 (Clontech)

Genotype: *MATa*, *trp*1-901, *leu*2-3, 112, *ura*3-52, *his*3-200, *gal*4Δ, *gal*80Δ, *LYS2::GAL1*$_{UAS}$*-GAL1*$_{TATA}$*-HIS3, MEL1 GAL2*$_{UAS}$*-GAL2*$_{TATA}$*-ADE2*, *URA3::MEL1*$_{UAS}$*-MEL1-*$_{TATA}$*-lacZ*

Reporter genes: *HIS3, ADE2, lacZ, MEL1*;

Transformation markers: *trp*1, *leu*2

Y187 (Clontech)

Genotype: *MAT*, *ura*3-52, *his*3-200, *ade*2-101, *trp*1-901, *leu*2-3, 112, *gal*4Δ, *met*$^{-}$, *gal*80Δ, *URA3::GAL1*$_{UAS}$*-GAL1*$_{TATA}$*-lacZ, MEL1*

Reporter genes: *lacZ, MEL1*;

Transformation markers: *trp*1, *leu*2

2.1.5 Plant material and growth conditions

All mutants described here were derived from *Arabidopsis thaliana* ecotype Columbia-0 (wild-type). Arabidopsis seeds were stratified for 4 days in 4°C before sowing on soil containing a mixture of substrate and vermiculite (3:1). Plants were grown under controlled environmental conditions (22°C and 50% RH) and 150 E·m-2·s-1 light (fluorescent Sylvania F72T12 cool-white light [75%] and incandescent Sylvania 100-W lamps [25%]) for either 16 h followed by 8 h of darkness (long days, LD) or 8 h followed by 16 h of darkness (short days, SD). Table 2-2 listed all mutants involved in this study. Transgenic plants generated during this study are listed in *Appendix A*.

Mutants	Gene of interest	Genotyping	PCR products
SALK_013458	At5g54510 (DFL1)	*wt* (ZY34/ZY35)	0.7 kb
		mutants (ZY36/ZY35)	0.7 kb
GABI-KAT 246A02	At3g09970 (CAP)	*wt* (ZY37/ZY38)	0.5 kb
		mutant (ZY39/ZY38)	0.5 kb
GABI-KAT 621B04	At3g09960 (CAPH)	*wt* (ZY46/ZY47)	0.8 kb
		mutant (ZY46/ZY39)	0.8 kb

Mutants	Gene of interest	Genotyping	PCR products
spl8-1	*At1g02065 (SPL8)*	*SPL8* (ZY67/ZY66)	0.8 kb
		spl8-1 (GC326/GC577)	0.2 kb
spl8-2		*SPL8* (ZY67/ZY66)	0.8 kb
		NA	
spl8-3		*SPL8* (ZY67/ZY66)	0.8 kb
		spl8-3 (GC577/GC637)	0.3 kb
spy-3	*SPINDLY*	determined by phenotype	
GABI-KAT 513C06	*GA1*	determined by phenotype	

Table 2-2: Mutants involved in this study. NA: not applicable.

2.1.6 Vectors

Vector	Selection Marker (final conc.)	Source
pGJ2171	Spectinomycin (75 mg/l, bacteria)/Basta (0.1%, plant)	G. Jach, MPIZ
pGJ2148	Spectinomycin (75 mg/l, bacteria)/Basta (0.1%, plant)	
pGBKT7	Kanamycin (50 mg/l, bacteria)/-Trp (yeast)	Clontech
pGADT7	Ampicillin (100 mg/l, bacteria)/-Leu (yeast)	
pRB504	Ampicillin (100 mg/l, bacteria)	R. Birkenbihl, MPIZ
pRB521	Ampicillin (100 mg/l, bacteria)	
pRB522	Ampicillin (100 mg/l, bacteria)	
pRB536	Ampicillin (100 mg/l, bacteria)	

Table 2-3: Vector backbones used in this study, including plant binary vectors pGJ2171, pGJ2148, protein expression vectors pRB504, pRB521, pRB522, pRB536, and yeast two hybrid vectors pGBKT7, pGADT7. All constructs generated from these vector backbones are listed in *Appendix B*.

2.1.7 Oligonucleotides

Oligonucleotides were synthesized by Invitrogen (The Netherlands). All oligonucleotides used in this study were listed in *Appendix C*.

2.1.8 Enzymes

Restriction enzymes were purchased from New England Biolab (USA) and Roche (Mannheim). T_4 DNA ligase and Klenow were purchased from

Roche (Mannheim). Taq polymerases were purchased from peqLab (Germany), either pwoTaq for cloning or normal Taq for genotyping. Reverse transcriptase Superscript II was purchased from Invitrogen (USA), and one-step RT-PCR reastions were conducted with Qiagen One-step RT-PCR kit (Valencia, CA). Lyticase used in yeast studies was from Sigma (USA). All enzymatic reactions were performed according to the manufacturers' recommendation.

2.1.9 Chemicals

Chemicals were purchased from the following biotech companies: BioRad (USA), Clontech (Heidelberg), Difco Lab (USA), Duchefa (The Netherlands), Invitrogen (The Netherlands), MBI Fermentas (Heidelberg), Merck (Darmstadt), Pharmacia (USA), Promega (Heidelberg), Roche (Mannheim) and Sigma (München). Nylon membrane used in Western blot was provided by Amersham-Buchler (Braunschweig). Radioisotope (α^{32}P-dCTP) was purchased from Hartmann Analytics (Braunschweig). All antibiotics were produced by Duchefa (The Netherlands). DNA gel purification and PCR product purification were performed using NucleoSpin® Column (Macherey-Nagel, Germany).

2.1.10 Media

Unless otherwise indicated, all the media were sterilized by autoclaving at 121°C for 20 min. For the addition of antibiotics and other heat-liable components, the solutions or media were first cooled down to 55°C.

LB (Lauria Bertani) Broth

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

Agar plates: 1.5-2% agar was added to the above broth.

SOC-Medium (100 ml)

Bacto -tryptone	2.0 g
Bacto -yeast extract	0.5 g

1 M NaCl	1 ml
1 M KCl	0.25 ml
2 M Mg^{2+} stock	1 ml
2 M glucose, filter-sterilized	1 ml

Add tryptone, yeast extract, NaCl and KCl to 97 ml distilled water. Autoclave and cool to room temperature. Just before each use, add 2 M Mg^{2+} stock and 2 M glucose, each to a final concentration of 20 mM.

YEB-medium

Gibco beef extract	5 g/l
Bacto-yeast extract	1 g/l
Bacto-peptone	1 g/l
Sucrose	5 g/l

Adjust pH to 7.4. After autoclaving, add 2 ml 1 M $MgSO_4$. For agar plates: 1.5-2% agar was added before autoclaving.

IM (infiltration medium)

1/2 MS salts (micro and macro)	2.205 g/l
1/2 x B5 vitamins	50 µl/l
Sucrose	50 g/l
Benzylaminopurine (BAP)	10 µl/l of 1 mg/ml stock
Surfactant SILWET L-77	0.005% v/v
pH 5.7 (KOH)	

GM (Germination medium)

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

YSD medium (1 L, pH 5.8)

Yeast Nitrogen Base	6.7 g
Agar (for plate only)	20 g
Drop-out solution (10X)	100 ml
40% glucose	50 ml
H_2O	850 ml

Allow medium to cool to ~ 55°C before adding 3AT or additional amino acids.

YPD (1 L, pH 6.5)

Peptone	20 g
Yeast extract	10 g
Agar (for plate only)	20 g
40% glucose stock	50 ml
H_2O	950 ml

YPDA

Add to 1 L of YPD 15 ml of 0.2% Adenine hemisulfate (final concentration 0.003%).

2.1.11 Buffers and solutions

2.1.11.1 General buffers and solutions

TE (Tris/EDTA) buffer

10 mM Tris/HCl (pH 8.0 or 7.5)

1 mM EDTA (pH 8.0) in H_2O

Tris/HCl (1 M)

Tris-Base	121 g
H_2O	1000 ml

Dissolve 121 g Tris base in 800 ml, adjust to desired pH with concentrated HCl. Adjust volume to 1 L with H_2O.

EDTA stock (0.5 M, pH 8.0)

EDTA	186.1 g
H_2O	1000 ml

Dissolve 186.1 g EDTA in 700 ml water, adjust pH to 8.0 with 10 M NaOH (~50 ml; add slowly), add water up to 1 L. Filter sterilize.

Sodium phosphate buffer (0.1 M)

Solution A: 27.6 g $NaH_2PO_4 \cdot H_2O$ per L (0.2 M final) in water.

Solution B: 53.65 g $Na_2HPO_4 \cdot 7H_2O$ per L (0.2 M final) in water.

Mix the different volumes of solutions A and B to 100 ml for desired pH, then dilute with water to 200 ml.

Potassium phosphate buffer (0.1 M)

Solution A: 27.2 g KH_2PO_4 per L (0.2 M final) in water.

Solution B: 34.8 g K_2HPO_4 per L (0.2 M final) in water.

Mix the different volumes of solutions A and B to 100 ml for desired pH, then dilute with water to 200 ml.

Ethidium bromide stock (10 mg/ml)

Ethidium bromide	0.2 g
H_2O	20 ml

Store at 4°C in the dark or in a foil-wrapped bottle. Do not sterilize.

TBE (Tris/borate/EDTA) buffer (10x)

Tris base	108 g
Boric acid	55 g
H_2O	960 ml
0.5 M EDTA (pH 8.0)	40 ml

DNA Gel loading buffer (6x)

Bromphenol blue	0.25%(w/v)
Xylene cyanol FF	0.25%(w/v)
gGlycerol	30%(v/v)

Store at 4°C (room temperature if Ficoll is used).

2.1.11.2 Buffers for bacteria DNA manipulation

TELT (*E.coli* DNA extraction buffer)

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

Lysozyme solution

Lysozyme	10 mg/ml
Tris/HCl pH7.5	10 mM
EDTA	0.1 mM

PIPES (0.5M)

PIPES 15.1g

Dissolve in 80 ml water, adjust pH to 6.7 with 5 M KOH, and to final volume 100 ml, filter sterilize then divide into aliquots and store in -20°C

Inoue transformation buffer

$MnCl_2 \cdot 4H_2O$	10.88 g
$CaCl_2 \cdot 2H_2O$	2.20 g
KCl	18.65 g
0.5 M PIPES	20 ml

Adjust volume to 1 L, filter sterilize, store in -20°C

2.1.11.3 Buffers for plant DNA manipulation

Plant genomic DNA extraction buffer (for PCR)

Tris/HCl pH 7.5	200 mM
NaCl	250 mM
EDTA	25 mM
SDS	0.5%

2.1.11.4 Buffers for plant RNA manipulation

10 x RNA gel buffer

MOPS	42 g/l
NaOAc	4.1 g/l
EDTA	3.7 g/l

Adjust pH to 7.0 with NaOH. Do not autoclave. Store at 4°C in the dark

RNA sample buffer

Deionized formamid	700 µl
35% formaldehyde	270 µl
10 x RNA gel buffer	30 µl, freshly prepare

RNA loading buffer

Ficoll	25%
EDTA	100 mM
Bromophenol blue	0.25% (w/v)

2.1.11.5 Buffers for western blot

Protein extraction buffer I

Tris/HCl pH 6.5	50 mM

EDTA	10 mM
Triton X-100	0.2%
KCl	200 mM
□-ME	0.1%

Protein extraction buffer II

Tris/HCl pH 6.8	150 mM
EDTA	6 mM
SDS	3%
□-ME	3%
Glycerol	24%
Bromophenol blue	0.075%

Special buffer

Freshly mix equal volume of protein extraction buffer I and II

Western transfer buffer (2L)

Tris	11.6 g
glycine	58 g
SDS	2 g
Methanol	400 ml

Add water up to 2 L

PBS (1L)

NaCl	8 g
KCl	0.2 g
Na_2HPO_4	1.44 g
KH_2PO_4	0.24 g in 800 ml water

Adjust pH with HCl to 7.4, add water up to 1 Liter, autoclave.

PBST

PBS + 0.1% Tween 20

Blocking solution (50ml)

PBST	25 ml
Transfer buffer	25 ml
Non-fat milk powder	2.5 g

2.1.11.6 Buffers for yeast manipulation

Li/TE (30 ml)

10 x LiAc	3 ml
10 x TE	3 ml
H_2O	24 ml

PEG/Li/TE (30 ml)

50% PEG	24 ml
10 x LiAc	3 ml
10 x TE	3 ml

Sorbitol solution

Sorbitol	1 M
EDTA	100 mM
□-ME	14 mM

2.1.11.7 Solution for anther histology assay

Araldite-mix

Araldite	5 ml
DDSA (pre-warmed at 60°C)	5.5 ml
BDMA	180 □l
Dibutylphtalat	250 □l

Gently mix in 15 ml Falcon tube, avoid bubbles.

2.2 Methods

2.2.1 DNA sequencing and sequence analysis

DNA sequences were determined by the MPIZ DNA core facility on Applied Biosystems (Weiterstadt, Germany) Abi Prism 377 and 3700 sequencers using BigDye terminator chemistry. Premixed reagents were from Applied Biosystems. DNA and protein sequence analysis was performed using the MacVector program (Oxford Molecular Group). In the assay of putative SPL8 interactors, prey sequences determined from the PCR products were "BLASTed" against the GenBank database (http://www.ncbi.nlm.nih.gov/BLAST/) and after identification annotated according to the MIPS (http://mips.gsf.de/proj/thal/db/search/search_frame.html) and TIGR (http://www.tigr.org/tdb/e2k1/ath1/LocusNameSearch.shtml) Arabidopsis sequence databases.

2.2.2 Plasmid construction

Constructs used in the study, including those in trangenic analysis, subcellular localization assay and those in yeast two hybrid assays are listed below.

pZY101-pGJ2148-*SPL8* genomic fragment

A 4.8 Kb *SPL8* genomic fragment was digested from BAC clone T7I23.32, Klenow filled, then inserted into pGJ2148/*Sma*I.

pZY102 (pGJ2171-*SPL8*)

To make *SPL8* overexpression constructs, *SPL8* entire coding sequence was amplified from vector pRB504 with primers ZY06 and ZY08 to introduce the cloning sites *Nco*I and *Xba*I at the 5' and 3' termini respectively. Amplification was followed by enzymatic digestion, and the DNA fragment, purified using NucleoSpin® Column (Macherey-Nagel, Germany) according to

manufacturer's recommendation, was subcloned in binary vector pGJ2171 which carries two copies of the Cauliflower Mosaic Virus 35S promoter.

pZY103 (pGJ2171-*SPL8 S63A*)

Constructed in the same way as pZY102, except that the template used for subcloning was pRB521.

pZY104 (pGJ2171-*SPL8 S63D*)

Constructed in the same way as pZY102, except that the template used for subcloning was pRB522.

pZY105 (pGJ2171-*SPL8 S49A*)

Constructed in the same way as pZY102, except that the template used for subcloning was pRB536.

pZY107 (pGJ2171-*SPL8:dGFP*)

To make GFP fusion constructs for subcellular localization assays, a two-step cloning method was used. *SPL8 CDS* was first amplified from vector pRB504 with primers ZY06 and ZY07 to introduce *Nco*I and *Afl*III sites. Amplification was followed by digestion, and the DNA fragment was subcloned in binary vector pGJ619, which has two copied of GFP at the C terminus. The *SPL8-GFP* fragment was then digested with *Nco*I and inserted into pGJ2171.

pZY108 (pGJ2171-*SPL8 S63A:dGFP*)

Constructed in the same way as pZY107, except that the template used for subcloning was pRB521.

pZY109 (pGJ2171-*SPL8 S63D:dGFP*)

Constructed in the same way as pZY107, except that the template used for subcloning was pRB522.

pZY110 (pGJ2171-*SPL8 S49A:dGFP*)

Constructed in the same way as pZY107, except that the template used for subcloning was pRB536.

pZY112 (pGJ2171-*SBP-box* of *SPL8*)

SBP-box of *SPL8* was PCR amplified with primer pair ZY11 and ZY12 from pRB504 to introduce the cloning sites *Nco*I and *Xba*I at the 5' and 3' termini respectively. Amplification was followed by enzymatic digestion, and the DNA fragment was subcloned in binary vector pGJ2171.

pZY113 (pGBKT7-*SPL8*Δ*nt1-535*)

SPL8 (Δ*nt1-535*) was amplified with primer pair ZY15 and ZY14 from pRB504 to introduce the cloning sites *Nde*I and *Xma*I at the 5' and 3' terminal respectively. Amplification was followed by enzymatic digestion, and digested DNA fragment was subcloned in Y2H bait vector backbone pGBKT7.

pZY114 (pGBKT7-*SPL8*Δ*nt643-1002*)

pZY114 was made in the same way as pZY113, except using primer pair ZY13 and ZY21.

pZY115 (pGBKT7-*SPL8*)

pZY115 was made in the same way as pZY113, except using primer pair ZY13 and ZY14. pZY115 expresses wild-type full length SPL8.

pZY116 (pGBKT7-*SPL8*Δ*nt1-418*)

pZY116 was made in the same way as pZY113, except using primer pair ZY23 and ZY14.

pZY118 (pGBKT7-*SPL8nt418-643*)

pZY118 was made in the same way as pZY113, except using primer pair ZY23 and ZY21.

pZY119 (pGBKT7-*SPL8 S247A*)

pZY119 was made in the same way as pZY115, except using pRB521 as the template.

pZY120 (pGBKT7-*SPL8 S247D*)

pZY120 was made in the same way as pZY115, except using pRB522 as the template.

2.2.3 Bacteria manipulation

2.2.3.1 Competent *E.coli* preparation

One *E. coli* colony growing on LB medium was used to inoculate 25 ml SOB in 250 ml flask, and incubated at 37°C for 6 to 8 hr with 270 rpm shaking speed. 4 ml such culture was transferred into 250 ml SOB in 1 L flasks and incubated at 18°C for 13 to 15 hr till OD_{600} reached 0.55. The culture was transferred into a pre-chilled tube and put on ice for 10 min, followed with centrifugation at 4000 rpm 4°C for 10 min. Supernatant was removed and pellet was re-suspended in 80 ml ice-cold Inoue buffer followed with centrifugation at 4000 rpm 4°C for 10 min. Pellet was again re-suspended in 20 ml ice-cold Inoue buffer, and followed with centrifugation at 4000 rpm 4°C for 10 min. The resulting pellet was re-suspended in 20 ml ice-cold Inoue buffer (7.5% DMSO). Aliquots were quickly snap-freezed in liquid nitrogen and stored at -70°C. The competent cells then were used for heat-shock transformation.

2.2.3.2 *E.coli* plasmid preparation

Overnight *E.coli* cultures were centrifuged to remove the supernatant. The pellets were re-suspended in 200 □l TELT (20 □l Lysozyme), incubated at 95°C for 3 min, then at 4°C for 3 min. A 1 min full-speed centrifugation step was conducted after the incubation and slimy pellets were removed. The remaining solution was added with 100 □l isopropanol, and centrifuged at 4°C for 15 min. Supernatant was discarded and pellets washed with 200 □l 70%

ethanol. Briefly centrifuge to remove the excess liquid. The final pellets were solved in 50 μl TE added with RNase.

2.2.3.3 Competent Agrobacteria preparation

5 ml YEB was inoculated with 5 μl Agrobacteria stock and incubated for 2 days at 28°C. The resultant culture was transferred into 500 ml YEB and incubated at 28°C for 4 to 6 hr. When OD_{600} reached 0.3 to 0.5, it was transferred on ice to cool down. Culture was then centrifuged at 4°C 4200 rpm for 15 min, and pellet re-suspended with 100 ml ice-cold H_2O. This step was followed by three times cooling, centrifugation and re-suspension with 50 ml ice-cold H_2O, 5 ml ice-cold 10% glycerol, and 800 μl ice-cold 10% glycerol respectively. Aliquots were quickly snap-freezed in liquid nitrogen and stored at -70°C. The competent cells then were used for electroporation.

2.2.4 Plant transformation

Arabidopsis plants were transformed using the floral-dip method (Clough and Bent, 1998). Transgenic plants were selected on soil by spraying seedlings with 0.1% BASTA (for pGJ2171 and pGJ2148 backbone vectors), and further confirmed by genotyping with bar-specific primer pair and 35S promoter/terminator specific primer pair.

2.2.5 Plant genomic DNA extraction for PCR

For genotyping transgenic plants or mutant plants, Arabidopsis leaves were collected, and DNA was isolated using a method adapted from Edwards et al. (1991). Briefly, around 100 mg leaves was grinded in 400 μl extraction buffer, followed with a vortex and centrifugation step. 300 μl supernatant was transferred into a tube pre-added with 300 μl isopropanol. The mixture was incubated at RT for 2 min and then centrifuged at 13000 rpm for 5 min. The resultant pellet was washed with 70% ethanol and dried. 50 to 100 μl TE was added to dissolve the DNA pellet.

2.2.6 Standard PCR program

PCRs were performed using MJ Research PTC-200 (Biozyme) with the following PCR programs:

1. 94°C 2 min
2. 94°C 40 sec
3. 55°C - 65°C 40 sec
4. 72°C 1 min
5. go to 2 for 28-32 cycles
6. 72°C 10 min

2.2.7 Plant total RNA extraction

Arabidopsis total RNAs were isolated from plant materials using the Qiagen RNeasy kit (Valencia, CA) according to the manufacturer's protocol Plant+Fungi. On-Column DNase digestions were performed during the RNA extraction procedure according to the manufacturer's protocol using DNase kit (Valencia, CA).

2.2.8 Semi-quantitative RT-PCR

Semi-quantitative reverse transcriptase–mediated polymerase chain reactions (sqRT-PCR) were performed either in a one-step way using Qiagen One-Step RT-PCR kit, or in a two-step way using Invitrogen Superscript II reverse transcriptase kit. Oligonucleotides used for RT-PCR reactions were listed.

gene of interest	RT-PCR primer pair	PCR product size (kb)
SPL8	ZY67+ZY66	0.83
RAN3	GC709+GC710	0.53
GA5	ZY78+ZY79	0.75
RGL2	ZY89+ZY90	0.3

Table 2-4. RT-PCR primer pairs.

Three-step cycling conditions were as follows: 24-32 PCR cycles at 94°C for 40 sec, 60°C for 40 sec, and 72°C for 50 sec. After amplification, the RT-PCR products were loaded on 1.5% agarose gel, scanned with Typhoon

8600 PhosphorImag (Amersham Biosciences), and quantified using ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

2.2.9 Anther histology

Embedding of floral buds was done with Epoxy resin kit (Agar Scientific Ltd, USA). Floral buds ranging from 0.3 mm to 1.5 mm in length were collected and put individually into 1.5 ml Eppendorf tube added with 100 µl fixative. 30 min vaccum was applied to remove the air bubbles trapped inside the tissues. Ethanol series were applied to remove the fixative, 10%, 30%, 50%, 70%, 90%, 2 times 100%, 1 h for each treatment on ice. The embedding steps with Araldite-mix were described by Sorensen and coworkers (2002). Embedded floral buds were cut into 0.5 µm sections using Reichert Ultramicrotome (Leica, Germany). Sections were stained in 0.1% toluidine blue (Chroma Gesellshaft Shaud, Germany), coverslipped with Entelan (Merck, Germany).

2.2.10 Pollen viability assay

For pollen viability test, pollen of wild-type opening flowers were stained with fluorescein diacetate (FDA, stock solution 20 mg/ml in acetone, 100 x dilution in 7% sucrose) for 30 min at room temperature, then rinsed with 7% sucrose (w/v). Anthers of *35S::SPL8* transgenic plants were crashed to release pollen, which was treated in the same way as wild-type. Stained pollen was photographed with blue filter (excitation filter BP 485, chromatic beam splitter FT 510, barrier filter 515 to 565).

2.2.11 Microscopy imaging

For light and fluorescent microscopy, a Leica binocular microscope (Wetzlar, Germany) equipped with a digital camera was used to photograph cotyledons, inflorescences, flowers and anthers. A Zeiss Axiphot Fluorescence microscope (Jena, Germany) mounted with a JVC KV-F70 digital camera was used to photograph the anther sections. Confocal

microscopy was conducted with Leica TCS 4D confocal laser-scanning microscope (Wetzlar, Germany).

For scanning electron microscopy, freshly prepared materials were mounted on aluminum specimen stubs using Tissue-Tek OCT compound (Sakura Finetek, Tokyo, Japan) and immediately snap-freezed in liquid nitrogen. Samples were transferred subsequently to a Zeiss DSM 940 electron microscope (Jena, Germany) equipped with a cryo-chamber (Oxford Instruments). After sublimation of possible ice on their surfaces, samples were sputter-coated with gold and examined at an accelerating voltage of 5 kV at 15 mm. Digital photographic images were cropped and assembled using Adobe Photoshop 7.0 (Adobe Systems, Mountain View, CA). Color and contrast corrections were performed on entire images only.

2.2.12 Germination assays

Seeds were first washed in 98% ethanol for 4 min, then in 10% hydrochloride for 10 min. Finally wash with autoclaved distilled water three times to remove the remaining hydrochloride. Sterilized seeds were plated on germination media (GM) supplemented (where appropriate) with GA_3 (Sigma, USA) or PAC (Duchefa, The Netherlands) and put at 4°C 4 days for stratification. Because seed storage time has a great impact on its germination rate, the experiments were performed using seeds with similar storage duration from all genotypes. Germination was recorded 7 day after the plates were moved to the above-mentioned SD growth chamber. Cotyledon expansion was scored as germination. The percentage of germination and standard deviation for each genotype were determined from 6 trials with 60 to 100 seed per trial.

2.2.13 Root elongation assay

For root elongation assay, Arabidopsis seeds were sterilized in the same way as in germination assays. 9 DAG seedlings grown on germination media supplemented (where appropriate) with GA_3 (Sigma, USA) or PAC (Duchefa, The Netherlands) were placed in parallel and scanned at a

resolution of 600 dpi. Root length was quantified using the Measure Angels tool of the ImageJ software (http://rsb.info.nih.gov/uh/)

2.2.14 Hormone treatment

For GA_3 treatment on soil-grown plants, 100 μM GA_3 (Duchefa, the Netherlands) in 0.02% Tween-20 was sprayed twice per week starting from 2-week-old seedlings. 0.02% Tween-20 spray was used as mock treatment. For germination assay and root elongation measurement, GA_3 or PAC (Duchefa, the Netherlands) stock solutions in ethanol were added in germination media after autoclaving to certain final concentrations. 100 μM MeJA (Sigma, USA) were used to treat mature anthers of *35S::SPL8* transgenic plants for dehiscence assay as described by Zhu et al. (2004).

2.2.15 Yeast two hybrid screening

General protocols performed in yeast two hybrid screening can be found in Clontech yeast two hybrid handbooks (www.clontech.com). Some modified protocols used in the study are listed below.

2.2.15.1 Yeast small scale transformation

Colonies from one plate were used to inoculate 2 ml YPD medium and cultured at 30°C for 12 to 16 hr. 500 μl overnight culture was then used to inoculate 20 ml YPD. Until OD_{600} reached 0.2, the culture was centrifuged at RT 4000 rpm for 5 min, re-suspended in 25 ml water. It was centrifuged at RT 4000 rpm for 5 min, and re-suspended in 1.5 ml Li/TE in an Eppendorf tube. Centrifuge at full speed for 1 min and re-suspend the pellet in 500 μl Li/TE. The obtained solution was then divided into 5 aliquots in separate tubes and every tube was added sequentially with the following material: 1 μl bait DNA, 16 μl pre-boiled herring sperm DNA, 10 μl DMSO, and 600 μl PEG/Li/TE. Gently mix then shake at 30°C for 30 min. The mixture was incubated at 42°C for 15 min, centrifuged at 4000 rpm for 1 min. The pellet was re-suspended in 200 μl 0.9% NaCl, plated on appropriate selection YSD plates and incubated at 30°C for 2 to 3 days.

2.2.15.2 Optimum of mating conditions

Several AH109 colonies transformed with pZY115 were used for the assay. These colonies, together with AH109 transformed with empty BD vector, were inoculated on YSD plates with different nutrient deficiency, including

-Trp-His (with 0 mM, 4 mM, 6 mM, 8 mM, 12 mM 3AT)

-Trp-Ade

-Trp-Ade-His (with 0 mM or 4 mM 3AT)

The plates were incubated at 30°C for 3 to 10 days. Healthy growth was used as a sign of leaky reporter expression. 4 mM 3AT was determined to be the optimal concentration for SPL8 full length bait, enough to inhibit background and also not too high to be detrimental to yeast growth.

2.2.15.3 Yeast mating

Y187 colonies expressing SPL8 were inoculated in 50 ml YSD/-Trp and cultured at 30°C for 20 to 24 hr until OD_{600} reached 0.9. At the same time, AH109 colonies expressing empty AD were inoculated in 20 ml SD/-Leu.

The cultures were centrifuged at RT 800 rpm for 5 min to collect cells. The bait was re-suspended in 25 ml 2xYPDA (50 □g/ml Kan). The empty AD was re-suspended in 10 ml 2X YPDA (50 □g/ml Kan) together with 200 □l bait culture, and incubated at 30°C 45 rpm for 22-24 hr. The remaining bait culture was added with 150 □l cDNA library (hosted by AH109), and incubated at 30°C 45 rpm for 22-24 hr when zygotes were formed.

The mating mixture of the bait and cDNA preys were separately centrifuged at RT 4000 rpm for 10 min, and pellet was suspended with 40 to 50 ml 0.5X YPDA (50 □g/ml Kan):0.9% saline (1:1). The resultant culture suspension was plated on selective media 1 ml/plate, including SD/-Trp-Leu-His plus 4 mM 3AT, SD/-Trp-Leu-Ade plus 4 mM 3AT, and SD/-Trp-Leu-His-Ade plus 4 mM 3AT. One SD/-Trp-Leu plate was used for counting the mating

efficiency. Positive colonies appeared within 10 days. The mating mixture of empty AD and the bait was cultured on similar selective media as the control.

2.2.15.4 Yeast colony PCR

Yeast colonies grown on selective plates after mating were considered positive and colony PCRs were preformed with pGADT7-specific sequencing primers on these positive colonies. Yeast colony was picked with an Eppendorf tip and suspended into 6 μl Lyticase solution (5 units/μl in TE buffer, store at -20°C for up to 6 months, at 4°C for up to 2 months), incubated at 37°C for 10 min. 2 μl was used as template for normal PCR reaction.

3. Results

3.1 *SPL8*, a dual regulator acting in a subset of gibberellin-mediated developmental processes

3.1.1 Introduction

The phytohormone gibberellic acid and its derivatives (GAs) are important plant growth factors regulating many developmental processes, such as seed germination, rosette leaf expansion, stem and root elongation, floral induction and anther development (for review, see Richards et al., 2001; Fleet and Sun, 2005). The diverse and vital effects of GAs on plant development require an appropriate endogenous GA level and also a fine-tuned GA signal response. Accordingly, extensive studies have been conducted, mainly through genetic screens, to get a better understanding of the GA biosynthetic and signaling pathways (for review, see Hedden and Phillips, 2000; Olszewski et al., 2002).

In recent years, the GA biosynthetic pathway has become well understood and GA-biosynthetic genes have been cloned and characterized from many plant species (reviewed by Hedden and Phillips, 2000). Mutants defective in these genes, like *ga1-3*, showed germination failure, dwarfism, late flowering, and male sterility (Koornneef and Van der Veen, 1980). The phenotypic alterations of GA-deficient mutants can be rescued by exogenous application of GA.

Similar, if not more, efforts have been dedicated to comprehend GA signaling and some recent results have given rise to a generally supported derepressible-repression model (Peng et al., 1999; Cheng et al., 2004; Tyler et al., 2004; illustrated in Fig. 3-1).

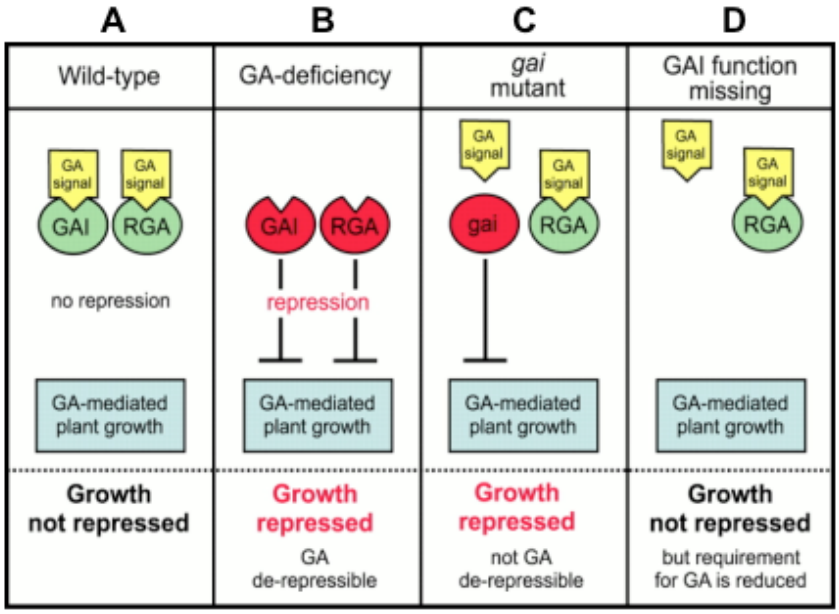

Figure 3-1. Illustration of the derepressible-repression model for GA action in plant development (picture adapted from Richards et al., 2001). In this model, GAs act through the repression of a group of negative regulators, the DELLA proteins.
(A) In wild-type, endogenous GAs derepresses the inhibitory effects of DELLA proteins, such as GAI and RGA, on GA-mediated plant growth.
(B) In the absence of endogenous GAs, DELLA proteins inhibit GA-mediated plant growth. Application of exogenous GAs derepresses this repression.
(C) Due to particular mutations, DELLA proteins become GA-insensitive and thus do not respond to GAs. Consequently, plant growth inhibition can not be released by the application of exogenous GAs.
(D) On the other hand, null mutations of DELLAs confer a largely GA-independent plant growth.

GA-signaling mutants supporting this model can be divided into two major classes according to their characteristic phenotypes. The first class consists of GA-insensitive mutants, which phenocopy GA deficient mutants but cannot be rescued by exogenously applied GAs. These mutants carry either dominant mutations in negative regulators or recessive mutations in positive regulators of the GA signaling cascade. For instance, the Arabidopsis *ga insensitive* (*gai*) mutant confers a dominant GA-insensitivity due to a 17 amino acid deletion within GAI, a DELLA protein negatively regulating GA-

mediated development (Peng et al., 1997). The deletion of the DELLA domain through the *gai-1* allele rendered GAI resistant to GA-dependent derepressing effects (Dill et al., 2004; Fu et al., 2004). Four more DELLA proteins have been identified sequentially in Arabidopsis, all showing similar functional mechanisms in GA-signaling (Silverstone et al., 1998; Lee et al., 2002; Wen and Chang, 2002; Tyler et al., 2004). The group of recessive GA-insensitive mutants is represented by *sleepy1* (*sly1*) (Steber et al., 1998). *SLEEPY1* encodes an F-box protein involved in GA-dependent DELLA protein degradation (McGinnis et al., 2003).

The second major class of GA-signaling mutants contain recessive mutations in negative regulators of GA signaling, which therefore, display a constitutive response to GAs. Seed germination of GA-constitutive response mutants is resistant against GA-biosythetic inhibitor paclobutrazol (PAC). Furthermore, these mutants display pale-green leaves, elongated stems and are male sterile. The *spindly* (*spy*) mutants belong to this group (Jacobsen and Olszewski, 1993). *SPINDLY* encodes a ser/thr O-linked N-acetylglucosamine transferase and has been proposed to function by competing for protein substrates with phosphatases and/or kinases (Filardo and Swain, 2003).

Although quite a few genes have been identified and the derepressible-repression model feasibly proposed, GA signaling as a whole is still elusive. Most GA signaling components characterized so far simultaneously cover a broad range of GA-regulated developmental processes, thus representing global GA response regulators. But bioactive GAs can have dramatically different effects on plant development depending on tissue type and developmental stage (Fleet and Sun, 2005). It is therefore reasonable to hypothesize that plants have evolved local regulatory networks to maintain appropriate GA levels and signaling in a specific-tissue and/or during a particular developmental stage. In contrast to our knowledge of the global regulators, much less is known about regulators affecting local GA signaling. Arabidopsis *SHORT INTERNODES* (*SHI*) might be one of such local regulators (Fridborg et al., 1999). SHI is a zinc finger protein, when

overexpressed, causing a delay in the floral transition and a reduction of the inflorescence stem. SHI is therefore proposed to act as a negative regulator primarily affecting stem elongation and flowering time (Fridborg et al., 1999).

It is reported here that the Arabidopsis SBP-domain protein SPL8 may represent such a local acting regulator. Likely acting as a transcription factor, SPL8 has previously been shown to be required for micro- and megasporogenesis as deduced from its semi-sterile mutant phenotype (Unte et al., 2003). In this study, *SPL8* gain-of-function transgenic lines were created and their pleiotropic phenotype suggested that SPL8 is involved in GA-signaling in a tissue-specific way. The studies provided evidence that *SPL8* acts locally in a subset of GA-mediated developmental processes.

3.1.2 The spatiotemporal expression of *SPL8* is critical for plant development

As previously reported by Unte et al. (2003), three *spl8* mutants were identified by their semi-sterile phenotype. Except for the defective micro- and megasporogenesis, *spl8* mutants also displayed shorter stamen filaments and a lower trichome density on their sepals, two traits regulated by GAs. Therefore, a possible role of *SPL8* in GA biosynthesis/signaling has been suggested (Unte et al., 2003).

In order to further investigate this possibility, *spl8* mutants were first treated with bioactive GAs. As no sign of rescue of the mutant phenotype could be observed, the possibility that SPL8 directly regulates GA biosynthesis can probably be excluded. To obtain additional clues on the possible role of SPL8 in GA-regulated development, an *SPL8* constitutive expression vector under the control of the strong and constitutive 35S-CaMV promoter was constructed and introduced into wild-type and *spl8* mutants. Proper transgenic expression was confirmed by RT-PCR (Fig. 3-2).

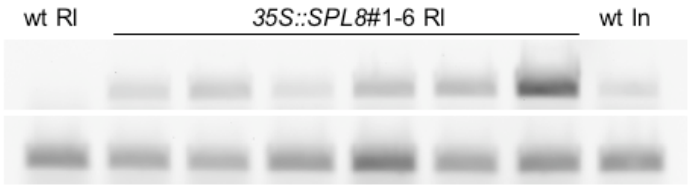

Figure 3-2. Confirmation of *SPL8* transgene expression. For the analysis by RT-PCR, RNA was isolated from seedlings 5 days after germination (DAG) and representing six independent *35S::SPL8* transgenic lines. A similar analysis of wild type and *spl8-1* mutant seedlings is shown for comparison. Rl, rosette leaves; In, inflorescence. Upper row, *SPL8*; below, *RAN3* (used as the internal control).

Constitutive *SPL8* expression in *spl8* mutants did not rescue their semi-sterile phenotype. In fact, the transgenic lines displayed an unexpected enhanced sterility, as also observed upon constitutive over-expression of *SPL8* in a wild-type background. In addition to sterility, the *SPL8* transgenic lines with either a *spl8* mutant or a wild-type background displayed a consistent and heritable pleiotropic phenotype, including reduced seed germination and root elongation, dwarfism, small rosettes, late flowering and a compact inflorescence.

In contrast to this dramatic gain-of-function phenotype, the sterility of *spl8* mutants transformed with *SPL8* under the control of its own promoter, as part of a 4.8 kb fragment spanning the *SPL8* genomic locus, was fully rescued, producing normal fertile plants without any obvious morphological defects (Fig. 3-3A). These contrasting results suggested that the spatiotemporal expression of *SPL8* is important for proper plant development. In wild-type plants, *SPL8* is predominantly expressed in the vegetative apex, inflorescence, and mature siliques. In seedlings and cauline leaves its expression is weak and, in other vegetative tissues *SPL8* transcription remains almost undetectable (Fig. 3-3B).

Figure 3-3. The spatiotemporal expression of *SPL8* is critical for plant development.
(A) *SPL8* under the control of its own promoter fully complemented its mutant phenotype, whereas *SPL8* under the control of the strong constitutive CaMV 35S promoter did not.
(B) Expression pattern of *SPL8* by semi-quantitative RT-PCR. Sl, seedlings 5 DAG; Rt, root; Rl, rosette leaves; Cl, cauline leaves; St, stem; Va, vegetative apex; In, inflorescence; Ms, mature siliques. *RAN3* was used as internal control.

3.1.3 Constitutive *SPL8* over-expression results in non-dehiscence of anthers

It is remarkable that loss-of-function as well as gain-of-function of *SPL8* both resulted in sterility. Unte et al. (2003) already showed that *SPL8* loss-of-function affected early stages of anther development starting with the initiation of the archesporial cells. Megasporogenesis was also found to be affected in *spl8* mutants such that some ovules degenerated at an early stage (Unte et al., 2003).

To determine whether the sterility was due to defects in anther development or ovule development, *35S::SPL8* transgenic plants and *spl8* mutants were manually pollinated with wild-type pollen. *spl8* mutants pollinated with wild-type pollen have siliques containing aborted ovules (Fig. 3-4A), indicating the defects of megaspore development as previously reported (Unte et al., 2003). Unlike *spl8* mutants, constitutive *SPL8* expression has no effect on ovule development, as deduced from the full seed set in the siliques after manual pollination of the transgenic flowers with wild-type pollen (Fig. 3-4B).

A statistical analysis was conducted regarding to the extent of sterility in both cases. Wild-type, *spl8-1*, and two *35S::SPL8* lines representing respectively a mildly (line 1) and a severely affected (line 3) phenotype, were grown under long day and short day conditions. The ratio of the number of sterile siliques to the total number of siliques on the main stem (around 30 at a late stage of inflorescence development) was determined. As shown in Figure 3-4C, under SD condition, about 40% of the siliques of the mild *35S::SPL8* line 1 is fertile, whereas in the severe line 3, less than 4% is fertile. In comparison, 80% of the *spl8-1* siliques are fertile. LD conditions reduced the fertility of all lines to similar degrees, resulting in around 20% fertile siliques produced by *35S::SPL8* line 1, and line 3 became almost completely sterile. Also the fertility of *spl8-1* is reduced in comparison to SD condition but it still produces 69% fertile siliques.

Stamen shapes at anther stage 9 were also compared since *spl8* mutants were reported to have abnormally short filaments and small anthers (Unte et al., 2003). Compared to that of wild type (Fig. 3-4D), stamens of *35S::SPL8* flowers were not obviously distinguishable (Fig. 3-4E), in dramatic contrast to the *spl8* stamens (Fig. 3-4F). These results demonstrated that anther defects might happen at a relatively late developmental stage in *35S::SPL8* plants.

To determine at which stage the anther developmental defects may appear, a histological analysis was performed. It turned out that the inability of

the anthers to dehisce causes the observed male sterility in *35S::SPL8* plants. At anther stage 11, wild-type tapetum starts to degenerate and endothecial cells enlarge (Fig. 3-5A, 5C). Anthers of *35S::SPL8* show comparable endothecial enlargement and degenerating tapetum (Fig. 3-5B, 5D). The only recognizable difference is that the middle layer, originally a single cell layer between endothecium and tapetum and disappearing at stage 8 in wild-type anthers, is still clearly seen in *35S::SPL8* anthers at stage 11 (Fig. 3-5D).

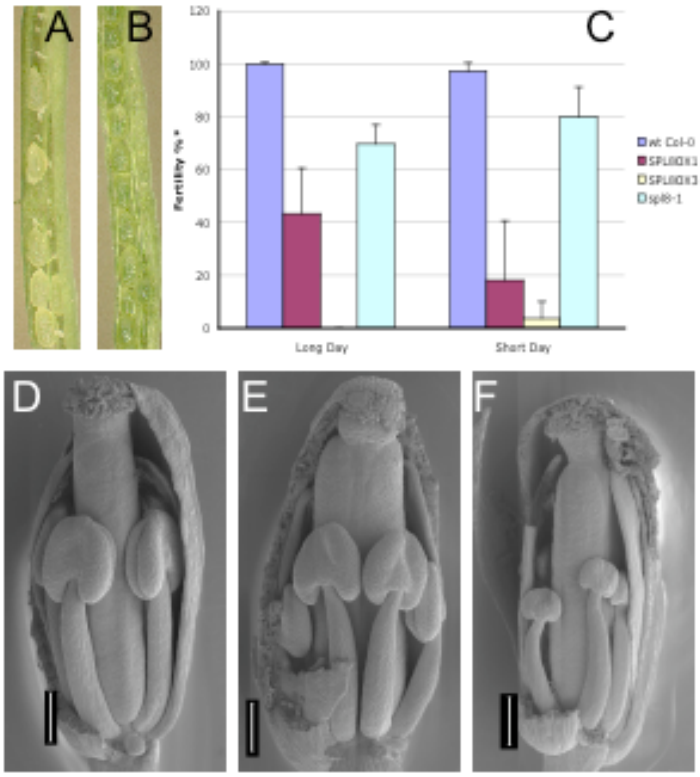

Figure 3-4. Constitutive *SPL8* expression results in male sterility.

(A) - (B) Siliques developed following manual pollination with wild-type pollen of an *spl8* flower (A), and of *35S::SPL8* flower (B).

(C) Statistical evaluation of fertility as recorded by the percentage of fertile siliques that developed under LD and SD conditions.

(D) - (F) SEM images of floral buds at proximately anther stage 10 after the completion of meiosis. (D) wild-type, (E) *35S::SPL8* flower (F) *spl8-1*. Scale bar = 200 µm.

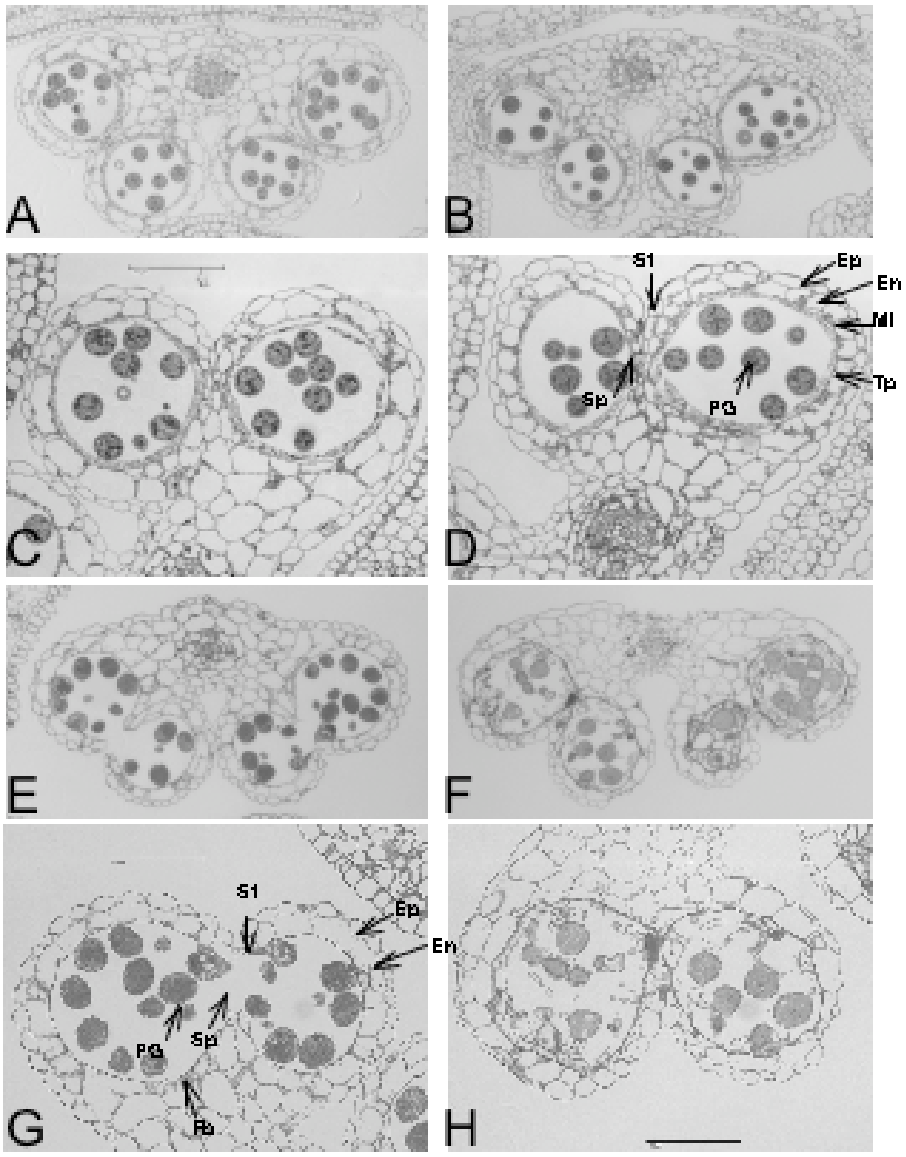

Figure 3-5. Constitutive *SPL8* expression results in non-dehiscent anthers. (A), (C), (E) and (G) wild type; (B), (D), (F) and (H) *35S::SPL8*.

(A) - (D) cross-sections through anthers at stage 11. (C) and (D) images taken at a higher magnification for (A) and (B) respectively.

(E) - (H) cross-sections through anthers at stage 13. (G) and (H) images taken at a higher magnification for (E) and (F) respectively.

En, endothecium; Ep, epidermal cell; Fb, fibrous bands; Ml, middle layer; PG, pollen grain; Sp, septum; St, stomium; Tp, tapetum.

Anther stage 13 is the stage where dehiscence happens (Sanders et al., 1999). The deposition of fibrous bands (lignification) in the wild-type connective and endothecial cells results in secondary wall thickening (Fig. 3-5E, 5G). A further enlargement of the endothecial cells together with a degenerating septum and stomium, results in rupture of the anther wall and to the release of the mature pollen grains (Fig. 3-5E, 5G). In comparison, deposition of fibrous bands in connective and endothecial cells of stage 13 *35S::SPL8* anthers is found to be largely absent (Fig. 3-5F, 5H). Instead, these cells accumulate some granular material (Fig. 3-5F, 5H). Furthermore, older *35S::SPL8* anthers show no signs of breakage of septum and stomium. As a result, pollen grains remain trapped inside the pollen sacs (Fig. 3-5F, 5H). The pollen sacs of *35S::SPL8* anthers at later stages are covered with a dark-staining layer on their inside and contain many small cellular structures of unknown origin besides pollen grains (Fig. 3-5F, 5H).

3.1.4 GAs might be responsible for anther dehiscence in *35S::SPL8*

Jasmonic acids (JAs) are known to be a major factor affecting anther dehiscence. Mutants defective in JA biosynthesis or signaling typically show a delayed anther dehiscence and pollen non-viability (reviewed by Scott et al., 2004). It was therefore tested if exogenously applied methyl jasmonate (MeJA) could rescue the non-anther dehiscence phenotype of *35S::SPL8* plants. However, no effects were observed, suggesting that constitutive over-expressing of *SPL8* does not affect JA biosynthesis. Furthermore, a pollen viability assay was performed on wild-type and *35S::SPL8* plants but again difference could not be detected suggesting that *35S::SPL8* plants are not disturbed in JA signaling (Fig. 3-6A, 6B).

GAs were indicated as a possible factor in anther dehiscence as studies in wheat and barley demonstrated that a GA overdose or constitutive response caused anther non-dehiscence (Colombo et al., 1996; Murray et al., 2003). Considering the anther defects and the reduction of fertility in both *SPL8* gain-of-function and loss-of-function, it was tempting to hypothesize

that anther non-dehiscence of *35S::SPL8* transgenic plants was due to a constitutive GA response.

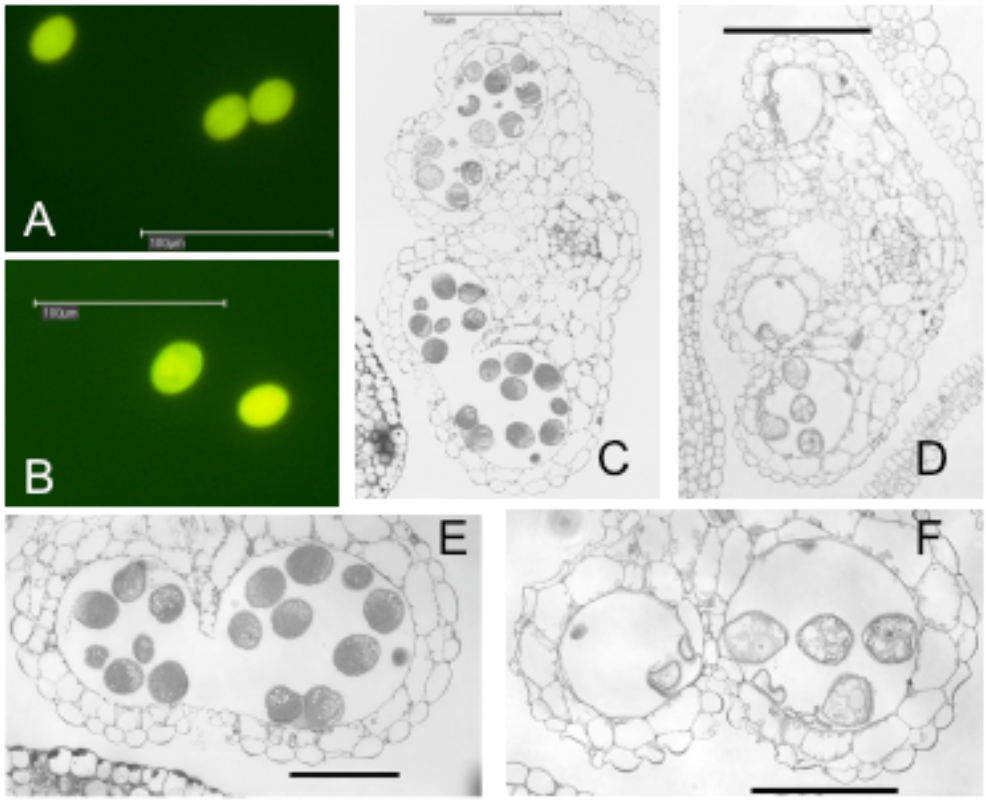

Figure 3-6. GAs might be responsible for the non-dehiscent phenotype of of *35S::SPL8* anthers.
(A) - (B) FDA viability staining of pollen. Yellow-green fluorescence indicates viable pollen (A) wild type, (B) *35S::SPL8*.
(C) - (F) cross-sections of anthers at stage 13. (C) and (E) wild-type, (D) and (F) *spy-3*. (E) and (F) images taken at a higher magnification for (C) and (D) respectively.
Scale bar equals: for (A) and (B) 100 µm, (C) and (D) 50 µm, for (E) and (F) 100 µm.

To find additional support for this idea, the anther histology of GA-constitutive response mutant *spy-3* (Jacoben and Olszewski, 1993) was examined first. Although the male sterility phenotype of *spy-3* has been known for more than ten years, the cause of its sterility has not been described in the literature. The histology of *spy-3* anthers before stage 11 could not be distinguished from those of wild type as judged on a microscopic analysis of transverse sections (data not shown). At stage 13 anther of *spy-3*

indeed showed non-dehiscence phenotype as reported in GA-overdosed wheat anther. As described before, the wild-type anther dehisces at stage 13 due to a series of histological events (Fig. 3-6C, 6E). While anthers of *spy-3* at stage 13, deposition of fibrous bands could be observed, albeit to a lesser extent than in wild-type. Septum and stomium did not degenerate, which probably was the major reason for the failure to dehisce (Fig. 3-6D, 6F).

3.1.5 The pleiotropic phenotype of 35S::*SPL8* transgenic plants resembles that of GA mutants

Besides male fertility, constitutive *SPL8* expression affected some other developmental processes, including seed germination, root and stem elongation, rosette leaf expansion, floral transition, inflorescence morphology, and trichome density on sepals.

Germination rate of *35S::SPL8* seeds was found to be reduced compared to wild-type seeds stored under identical conditions and for a same period of time. Five days after germination (DAG), wild-type seedlings showed fully opened, round cotyledons, *35S::SPL8* displayed narrower and darker-green cotyledons (Fig. 3-7A). Due to a prolonged growth along the proximal-distal axis, the final surface area of mature *35S::SPL8* cotyledons became comparable to that of wild-type (Table 3-1). The germination rate and cotyledon shape of *spl8* mutants were similar to those of wild type but *spl8* mutants showed an obvious increase in the surface area of their cotyledons (Table 3-1). Scanning electron micrographs, demonstrated that constitutive *SPL8* expression dramatically reduced the elongation and differentiation of epidermal cells on the adaxial side of cotyledons at 5 DAG. The stomatal cells of wild-type cotyledons at 5 DAG were fully differentiated with no meristemoids detectable anymore, and the pavement cells displayed a highly interlocking structure. In contrast, there were still ample undifferentiated meristemoids surrounding stomatal cells in *35S::SPL8* transgenic plants. The pavement cells of *35S::SPL8* plants were less complex shaped with no or a greatly reduced lobed structure (Fig. 3-7B), and much smaller than those of wild type (Table 3-1). Epidermal cells of *35S::SPL8* hypocotyls showed a

similar reduction in cell elongation (Fig. 3-7C) but no difference in hypocotyl length was observed between wild-type, *35S::SPL8* transgenic plants, and *spl8-1*. Constitutive *SPL8* expression reduced root elongation significantly. At 9 DAG, roots of *35S::SPL8* seedlings reached only half the length of wild-type. In contrast, *spl8* mutants revealed an enhanced root growth (Table 3-1).

Rosette leaf expansion in *35S::SPL8* transgenic plants was reduced in both the proximal-distal and medial-lateral axis, creating shorter and narrower rosette leaves (Fig. 3-7D). SEM images of the adaxial side of the tenth true leaf showed that the reduction in the leaf surface area of *35S::SPL8* transgenic plants was due to a reduction in cell elongation rather than cell number (Fig. 3-7E).

The observed delay in the onset of flowering in *35S::SPL8* plants varied from an additional 15 days under SD condition to 3 days under LD condition (Table 3-1). Remarkably, the number of rosette leaves produced by wild-type and *35S::SPL8* transgenic plants remained approximately the same, suggesting little change in relative developmental timing. The reduction in height of the *35S::SPL8* plants correlated with the degree of delay in flowering time (Table 3-1).

Also the Inflorescences of *35S::SPL8* transgenic plants differed from wild type such that they display a more compacted architecture due to a reduced elongation of the inflorescence stem. In this aspect, *spl8-1* did not differ from wild type (Fig. 3-8A). Floral organ shape was slightly changed by constitutive *SPL8* expression as displayed by the outward-bending sepals of *35S::SPL8* flowers (Fig. 3-8B). There were no other obvious changes regarding to the morphology of the floral organs. But consistent with the observed reduction in cell elongation during vegetative growth, epidermal cells of *35S::SPL8* transgenic petals and sepals were in general found to be smaller than those of wild type (Fig. 3-8C, 8D). Trichome density on the abaxial side of *35S::SPL8* transgenic sepals increased, as well as their complexity such that many displayed two or even three-branched structures (Fig. 3-8E). This contrasts to the majority of unbranched trichomes on wild

type sepals. Trichome density on *35S::SPL8* rosette leaves did not significantly differ from that of wild type.

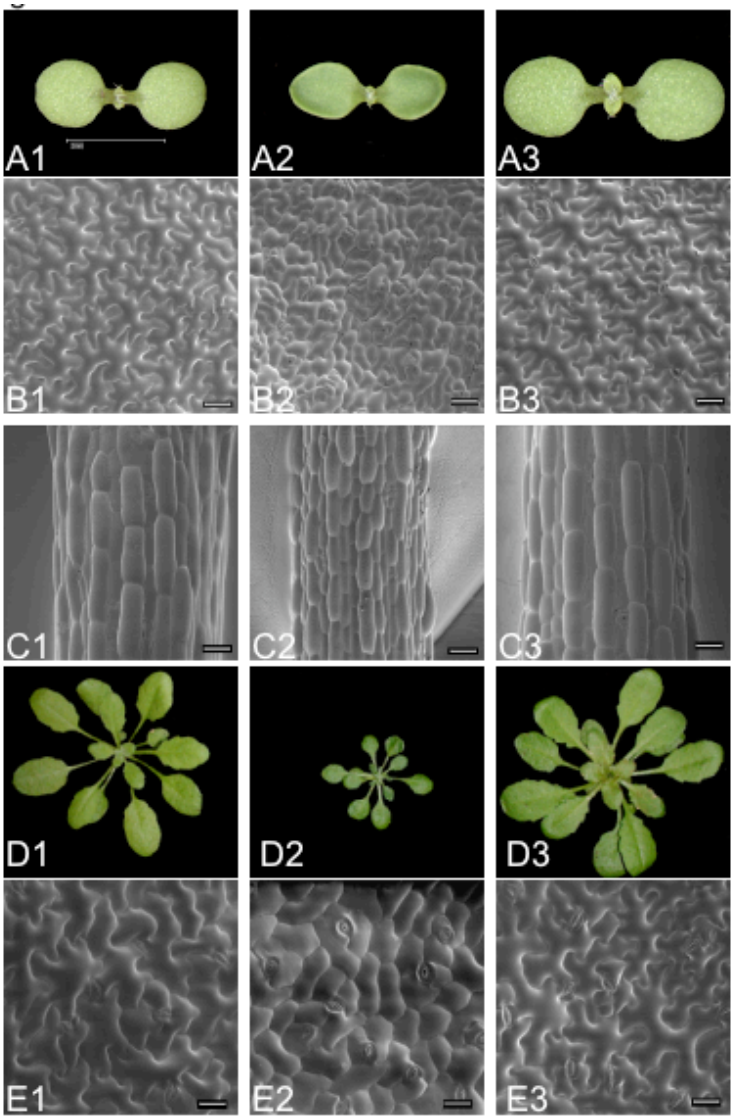

Figure 3-7. Constitutive *SPL8* expression results in a pleiotropic phenotype. 1, wild-type; 2, *35S::SPL8* transgenic plant; 3, *spl8-1* mutant.

(A) cotyledons 5 DAG.

(B) SEM images of the epidermis of cotyledons 5 DAG.

(C) SEM images of the epidermis of hypocotyls 5 DAG.

(D) plants at 30 DAG under SD conditions.

(E) SEM images of the epidermis of the tenth rosette leaf of plants 30 DAG under SD condition.

Scale bars equal: for (A) 2 mm, for (B) and (C) 50 μm, for (E) 20 μm.

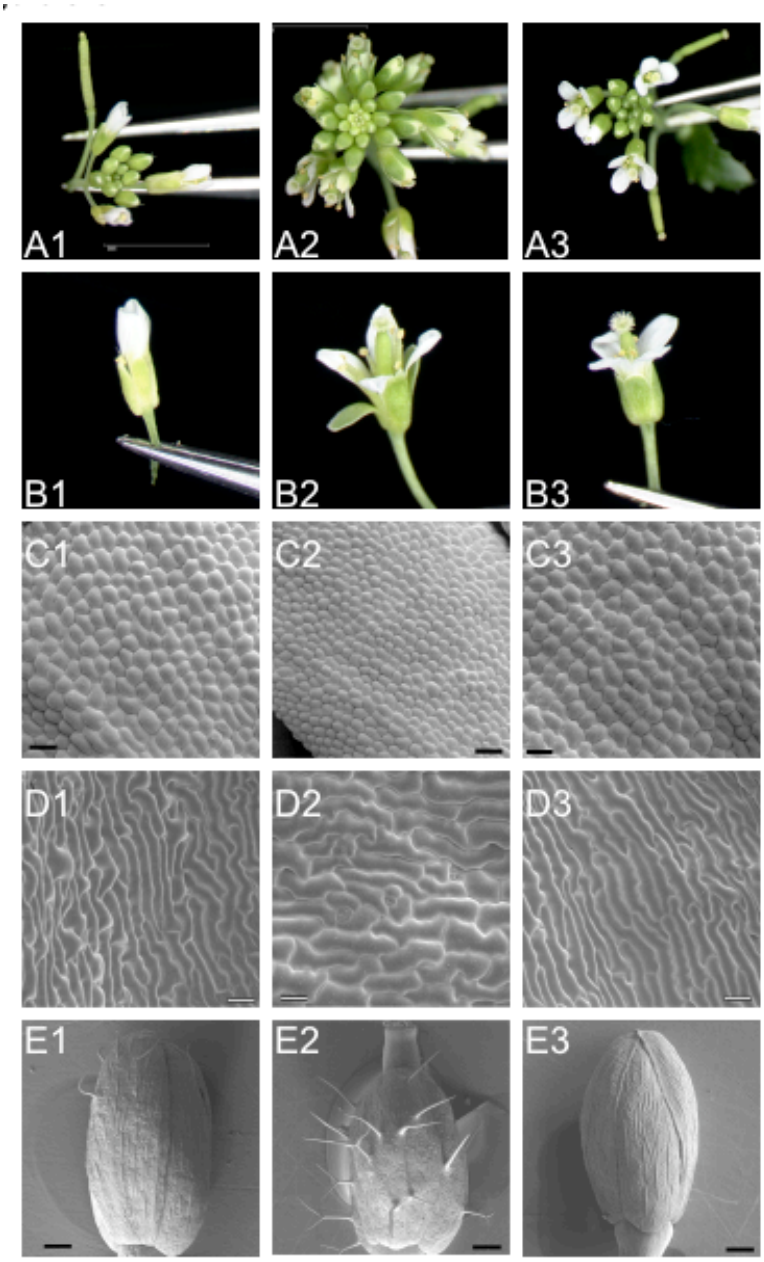

Figure 3-8. Constitutive *SPL8* expression results in a pleiotropic phenotype. 1, wild-type; 2, *35S::SPL8* transgenic plant; 3, *spl8-1* mutant.

(A) Inflorescences of plants grown under SD conditions.

(B) Mature flowers.

(C) SEM images of epidermal cells on the abaxial side of petals.

(D) SEM images of epidermal cells on the adaxial side of sepal.

(E) SEM images of floral buds showing altered density and branching of trichomes on the sepals.

Scale bar equals: for (C) and (D) 20 μm, for (E) 200 μm.

variables		**wild type**	***35S::SPL8* line 1**	***35S::SPL8* line 3**	***spl8-1***
Cotyledon surface area in mm^2(5 DAG/SD)		2.86 ± 0.42 (48)	2.59 ± 0.34 (47)	*ND	4.59 ± 0.69 (37)
Cotyledon pavement cell surface area in μm^2 (5 DAG/SD)		7649 ± 5190 (35)	1220 ± 690 (522)	*ND	6054 ± 4118 (63)
Hypocotyl length in mm (9 DAG/SD)		2.2 ± 0.2 (11)	2.2 ± 0.3 (15)	*ND	2.7 ± 0.8 (15)
root length in cm (9 DAG/SD)		1.26 ± 0.15 (11)	0.69 ± 0.19 (15)	*ND	2.0 ± 0.19 (15)
Flowering time in days	SD	72 ± 2.5 (54)	83 ± 2.9 (54)	86 ± 4.6 (51)	76.4 ± 3.9 (54)
	LD	36.4 ± 0.9 (50)	39.4 ± 1.5 (32)	42 ± 2.7 (37)	38 ± 1.2 (51)
Stem length in cm, LD		11.4 ± 0.95 (50)	9.9 ± 1.4 (32)	4.9 ± 2.2 (37)	11 ± 1.6 (51)

Table 3-1. Statistical analysis of features differing between *35S::SPL8* transgenic plants, wild-type and *spl8-1* mutant plants. *ND, not determined. Values represent averages; +/-, standard deviation, within bracket, number of measurement.

3.1.6 *SPL8* affects endogenous GA level/signaling in a spatiotemporal way

As *35S::SPL8* plants partly simulated GA-deficient mutants, it was tested if the *35S::SPL8* pleiotropic phenotype could be rescued through the exogenous application of GA. Several of the phenotypic alterations responded to the exogenous GA application such that in the transgenic lines stem length, rosette leaf size and flowering time became comparable to wild-type plants. This outcome strongly suggests that constitutive *SPL8* expression indeed reduced endogenous GA level in the affected organs and tissues. Regarding to trichome density, stamen filament elongation and anther development, *35S::SPL8* plants mimicked GA-constitutive response. Accordingly, no effects were observed on these features upon GA treatment of *35S::SPL8* plants.

A germination test was conducted to examine if the reduced germination rate of *35S::SPL8* can be rescued by GAs. Since there exist a few bioactive GAs that may function in different developmental processes, the first experiment was conducted to confirm that GA_3 is functional by counteracting the inhibitory effect of GA biosynthetic inhibitor PAC during wild-type and *spl8-1* seed germination (Fig. 3-9A).

The germination rate of *35S::SPL8* seeds on normal germination medium was found to be lower than those of wild type, and could not be rescued by the addition of GA_3 even at a concentration of 50 μM (Fig. 3-9B). This suggests that the germination failure of *35S::SPL8* transgenic plants is a result of GA-insensitivity rather than a reduced endogenous GA level. The effect of constitutive *SPL8* expression on root elongation also could not be compensated by the addition of GA_3 (Fig. 3-9C). In these aspects, constitutive *SPL8* expression conferred a certain GA-insensitivity in the seedling stage.

Considering the relatively higher PAC resistant germination showed by *spl8-1* (Fig. 3-9A), it was tempting to hypothesize that *SPL8* loss-of-function mutants might show the opposite effect, namely in displaying a constitutive GA response. Indeed, in contrast to *35S::SPL8* plants, the three available *spl8* allelic mutants turned out to be more resistant to PAC during seed germination than wild type (Fig. 3-9D). Whereas the germination rate of wild-type seeds decreased from 93% to 20% in the presence of 10 μM PAC, the germination rates of the *spl8* mutants remained well above 50%. Taken together, the results strongly suggest that an *SPL8* gain- or loss-of-function reduces or enhances GA signaling during seed germination respectively.

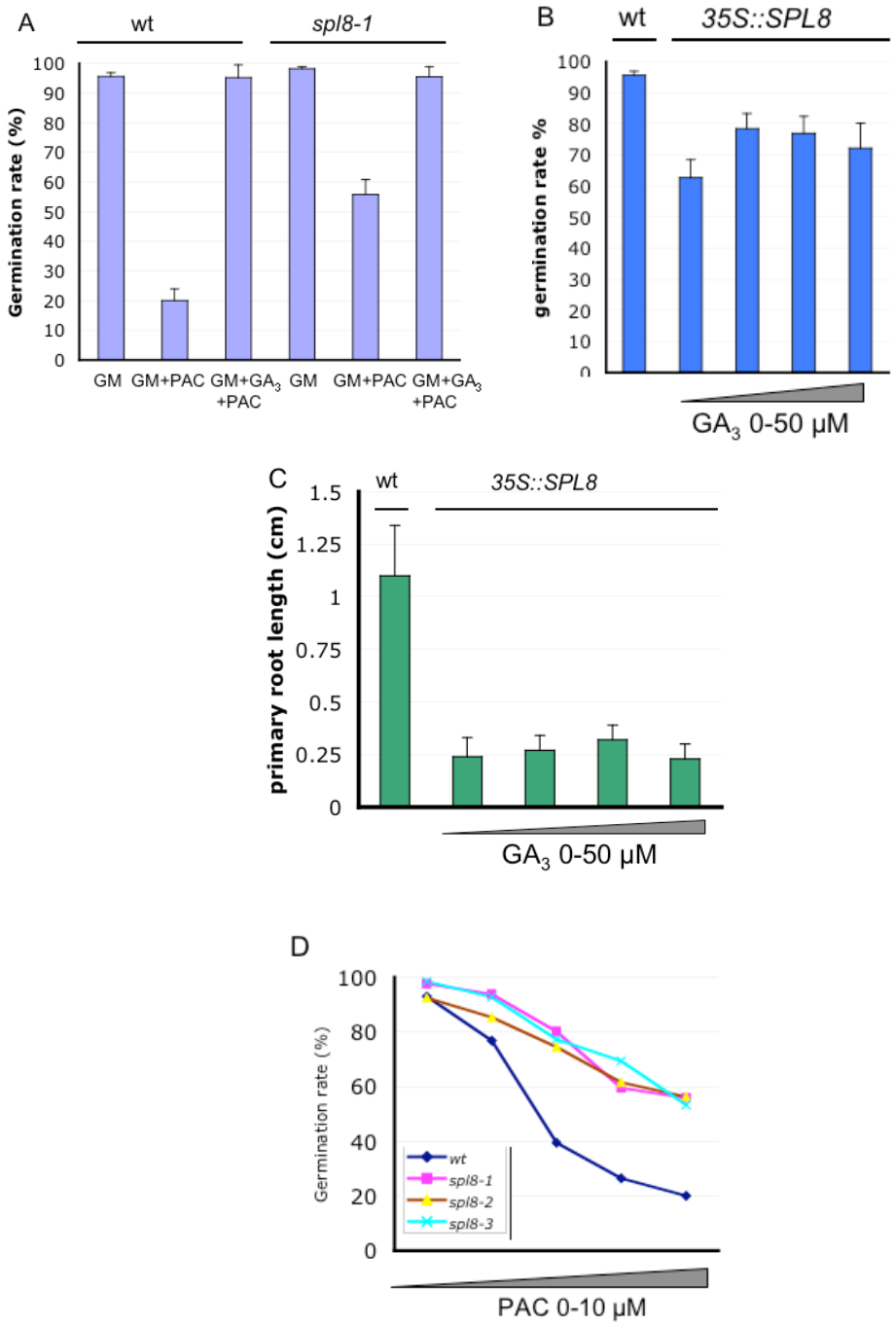

Figure 3-9. Germination and root elongation assays. Seed germination was measured at 5 DAG.

(A) GA_3 is functional during seed germination. This test also revealed that *spl8-1* has a higher resistance to the GA biosynthetic inhibitor PAC.

(B) Bioactive GA_3 can not rescue the low germination of *35S::SPL8* seeds.

(C) Bioactive GA_3 can not rescue the reduced root elongation of *35S::SPL8* seedlings.

(D) Seed germination of three *spl8* allelic mutants shows a higher resistance to PAC.

3.1.7 *SPL8* affects the expression levels of genes involved in GA biosynthesis/response

Alterations in the endogenous GA level/response can be reflected by changed transcript levels of GA biosynthetic/response genes. Arabidopsis *GA5* and *RGL2* are usually considered indicators for an altered GA level/response. *GA5* encodes a key GA biosynthetic enzyme and found to be controlled by GAs through a transcriptional feedback regulation (Xu et al., 1995; 1999). *RGL2* encodes a DELLA protein (Lee et al., 2002) and its levels are reduced by GAs after the completion of germination at seedling stage (Bassel et al., 2004).

As described above, *SPL8* gain- and loss-of-function displayed contrasting effects in GA-response during seed germination and seedling stage. The possibility that *SPL8* affects the endogenous GA level/response was therefore investigated by comparing transcript levels of *GA5* and *RGL2* in seedlings of wild-type, *35S::SPL8*, and *spl8*-1 all at 5 DAG. As shown in Figure 3-10A, seedlings of *35S::SPL8* transgenic plants have *RGL2* transcript levels of only half of that found in wild-type. On the contrary, the *RGL2* transcript level was found to be increased in *spl8-1*, in comparison to wild-type. *GA5* levels were reduced in both *spl8-1* and *35S::SPL8*. These results thus support the idea that *SPL8* exerts it effects during the seedling stage through altering the endogenous GA level/response.

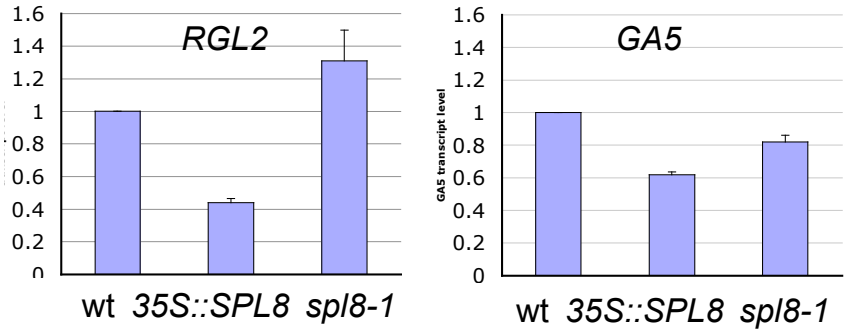

Figure 3-10. Genes involved in GA biosynthesis/signaling display altered transcript levels in response to an altered *SPL8* expression. *RAN3* was used as an internal control.

3.1.8 Discussion

GAs are important growth factors in plant development. The diverse effects of GA on plant development make it critical to maintain an appropriate endogenous GA level and a fine-tuned GA signaling. Most GA signaling components identified so far affect a full spectrum of GA regulated developmental processes, suggesting that they regulate relatively early events during GA signal transduction.

Except for these global regulators, there should also be a demand for local regulators, whose spatiotemporal expressions regulate GA level and/or response in selected tissue and during particular developmental stages. One such local functioning GA biosynthetic regulator, *DWARF AND DELAYED-FLOWERING1* (*DDF1*), has been characterized (Magome et al., 2004). Its dominant mutant *ddf1* showed a dwarf and delayed flowering phenotype but other GA-mediated developmental aspects such as germination, flower morphology and fertility remained unaffected. *DDF1* was found to be expressed mainly in vegetative tissues, which explains its locally restricted effects. Less is known about regulators affecting local GA signaling. Arabidopsis *SHORT INTERNODES* (*SHI*) might be such a local regulator (Fridborg et al., 1999) as overexpression of *SHI* causes mainly a shortening of the inflorescence stem and a delay in the floral transition.

In this project, the involvement of *SPL8* in GA-mediated developmental processes was investigated. Based on the experimental results obtained, it is proposed that *SPL8* acts in reproductive growth, seed germination, and root elongation as a local GA response regulator. Its role, i.e. as a negative or positive regulator, depends on the type of tissue. GA-deficiency/insensitivity leads to anther defects either post-meiotically or pre-meiotically, depending on the plant species investigated (Jacobsen and Olszewski, 1991; Izhaki et al., 2002; Huang et al., 2003; Cheng et al., 2004). Also the elongation of stamen filaments is disturbed in GA-deficient/insensitive mutants (Cheng et al., 2004) and trichome density responds to GA as a result of a GA-dependent upregulation of *GLABROUS1* (Perazza et al., 1998).

GA-constitutive response mutants, such as *spy-3*, display male sterility just as GA-deficient/insensitive mutants, but most probably due to a different mechanism (Jacobsen and Olszewski, 1993). A study by Colombo et al. (1996) revealed that excessive exogenous GAs can cause anther non-dehiscence in wheat and thereby male sterility. Male sterility due to the non-dehiscence of anthers was also observed by constitutively expressing *HvGAMYB* in barley (Murray et al., 2003). *HvGAMYB* has been identified in the barley aleurone layer as a positive regulator in GA signaling (Gubler et al., 1999), and found to be transcriptionally upregulated by GAs in anthers (Murray et al., 2003). Therefore, anther non-dehiscence by constitutive *HvGAMYB* expression is presumably the result of an enhanced GA response in barley anthers. The anther histology of *spy-3* described in this report shows that a GA-constitutive response results in anther non-dehiscence in Arabidopsis as well.

The reproductive abnormal phenotypes caused by a GA deficiency/insensitivity or a GA overdose/constitutive-response, correlate to a large extent with what could be observed in the phenotypes of *SPL8* loss- and gain-of-function, respectively. The involvement of *SPL8* in GA-mediated reproductive growth was first hinted by its mutant phenotype. *spl8* mutants are indistinguishable from wild-type during vegetative growth, which correlates with the absence of its expression in most vegetative tissues. During reproductive growth, *spl8* mutants show early anther developmental defects, shortened stamen filaments, and a reduced trichome density on sepals (Unte et al., 2003). In an opposite way, constitutive *SPL8* expression causes anther non-dehiscence, and high trichome density, indicating an enhanced GA signaling in anthers. Stamen filaments were unaffected in *35S::SPL8* plants.

The possible involvement of other phytohormones in the non-dehiscence of *35S::SPL8* transgenic plants was also taken into consideration. In Arabidopsis, JAs are major factors affecting anther dehiscence (reviewed by Scott et al., 2004). Mutants defective in JA biosynthesis such as *delayed dehiscence1*, *dad1* and *opr3* or in JA signaling like *coi1*, typically show

delayed anther dehiscence, shortening of stamen filaments and pollen non-viability (Sanders et al., 2000; Ishiguro et al., 2001; Stintzi and Browse, 2000; Xie et al., 1998). The phenotype of JA deficient mutants can be rescued by the exogenous application of JA while that of JA signaling mutants can not. The application of MeJA can not rescue the failure of anther dehiscence in *35S::SPL8* transgenic plants, excluding the possibility that *SPL8* is involved in JA biosynthesis. Furthermore, the non-dehiscence in *35S::SPL8* transgenic plants is also unlikely to be the result from a reduced JA signaling since constitutive *SPL8* expression does not affect pollen viability.

The effect of ethylene on anther development is mainly to synchronize pollen release with flower opening, while anther cell structure is not disturbed by ethylene-deficiency/insensitivity (Rieu et al., 2003). Therefore, a disturbed ethylene signal can not be the cause for the *35S::SPL8* phenotype.

Besides its role in reproductive growth, *SPL8* also affects two other developmental processes regulated by GAs, seed germination and root elongation. GAs promote these features by counteracting the inhibitory effect of DELLA proteins (Lee et al., 2002; Tyler et al., 2004; Fu et al., 2003). The GA-deficient mutant *ga1-3* can not germinate without exogenous GAs and root length is reduced (Koornneef and Van der Veen, 1980; Fu et al., 2003). In contrast, seed germination of GA-constitutive response mutants is PAC-resistant and these mutants can complement the root growth of *ga1-3* (Jacobsen and Olszweski, 1993; Fu et al., 2003; Tyler et al., 2004).

35S::SPL8 transgenic plants show a reduced seed germination rate and reduced root elongation. *spl8* mutants, on the contrary, display a higher PAC-resistant germination and an enhanced root elongation, both suggesting an elevated GA-signaling during the seedling stage. The negative regulation of *SPL8* in GA-mediated seed germination and root elongation is further supported by molecular data. *RGL2* and *GA5*, genes transcriptionally downregulated by endogenous GAs (Bassel et al., 2004; Xu et al., 1995; 1999), are downregulated in *35S::SPL8* transgenic seedlings. Ross (1994) hypothesized that in GA signaling mutants, those with an increased signal

transduction have a reduced level of active GA, while those with decreased signal transduction contain elevated levels of active GA. The feasibility of this hypothesis is now supported by a lot of data (Silverstone et al., 1998; Cowling et al., 1998). According to this hypothesis and given that *SPL8* regulates GA signaling in a negative way during the seedling stage, a reduced GA signaling by constitutive *SPL8* expression will result in increased endogenous GA level, and hence in a reduction of *RGL2* and *GA5* transcript levels as observed.

In *spl8* mutants the *RGL2* transcript level is increased, which also correlates with the hypothesis as a result of a reduced endogenous GA level due to the removal of a negative regulator. However, there is a discrepancy regarding to the transcript levels of *GA5* determined in *spl8*. Assuming that *SPL8* acts negatively in GA signaling, *spl8* mutants would have an enhanced GA signaling, leading reduced GA levels and accordingly high *GA5* transcription due to negative feedback control. One possible explanation for the fact that this could not be observed mutants is that the GA-dependent feedback regulation on *GA5* is disturbed *spl8* mutants. Similarly, Cowling et al. (1998) demonstrated that dominant GA-insensitive *gai* mutants contain high endogenous GA level and also a 4-fold higher *GA4* level, suggesting that *gai* mutants have a perturbed feedback regulation. A study by Meier et al. (2001) indicated that the GA-dependent negative feedback control on *GA5* is regulated by protein factors which may include a labile transcriptional repressor and/or factor. It is thus not unlikely that the GA-dependent negative feedback is also disturbed in the *spl8* loss-of-function mutant.

35S::SPL8 transgenic plants also display a pleiotropic phenotype during vegetative growth, including reduced rosette leaf expansion, stem elongation and delayed flowering. The effect of constitutive *SPL8* expression on these vegetative tissues can be set off by exogenously applied GAs, indicating a reduced endogenous GA level. Considering the spatiotemporal expression of *SPL8* and the absence of an obvious loss-of-function phenotype in these tissues, the pleiotropic phenotype during vegetative growth of *35S::SPL8* transgenic plants is probably due to a perturbed GA biosynthesis/signaling in response to ectopic *SPL8* misexpression.

In conclusion, the obtained experimental results indicate that *SPL8* plays a dual role in a subset of GA-mediated developmental processes. *SPL8* functions positively during GA-mediated anther development, but negatively in GA-mediated seed germination and root elongation (Fig. 3-11). Future efforts to find out the genetic interactions between *SPL8* and other GA-signaling components and to identify *SPL8* target genes will surely shed more light on the regulatory role of *SPL8* in GA-mediated plant development.

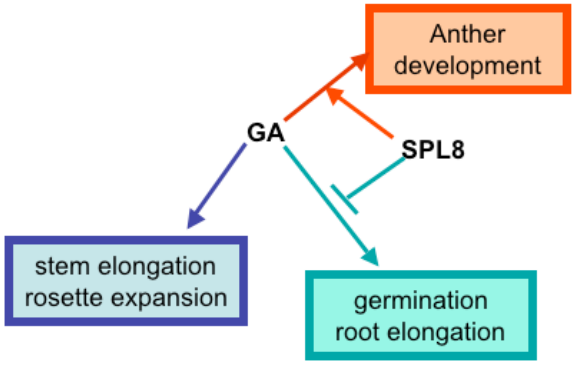

Figure 3-11. *SPL8* working model. *SPL8* acts negatively on GA-mediated seed germination and root elongation, but positively on GA-regulated anther development.

3.2 The subcellular localization of SPL8

3.2.1 Introduction

To fulfill their role in the regulation of nuclear gene transcription, transcription factors must become nuclear localized. Indeed, the maize SBP-domain protein LG1 was confirmed to be nuclear localized using a fluorescent protein fusion transiently expressed in a heterologous tissue (Moreno et al., 1997). The subcellular localization of Arabidopsis SPL8 has not been tested, although SBP-domain proteins contain a highly conserved bipartite nuclear localization signal (NLS), suggesting the probability of SBP-domain proteins being nuclear proteins.

The NLS of SBP-domain proteins is distinct in that it overlaps with a zinc-finger structure of DBD, the SBP-domain (Yamasaki et. al., 2004; Birkenbihl et al., 2005). Furthermore, the NLS was predicted to contain a potential phosphorylation site, an evolutionarily conserved serine residue (S63 in SPL8; Birkenbihl et al., 2005). Due to the sequence overlap, the phosphorylation status of S63 within the SBP-domain might affect zinc-dependent DNA binding as well as the nuclear localization of SPL8. Furthermore, phosphorylation on ser/thr residues is under the control of kinases/phosphatases whose actions depend on appropriate input signals. The existence of the phosphorylation site thus provides the possibility that the subcellular localization and DNA-binding of SPL8 respond to particular signal inputs. These special features would allow a multi-level regulation of SPL8 functionality.

3.2.2 Generation of SPL8-GFP fusion constructs

Several SPL8-GFP constructs were generated both to confirm that SPL8 is a nuclear protein and to investigate whether the highly conserved phosphorylation-potential of S63 may have effects on the subcellular distribution of SPL8. Point mutations were created to generate S63A and S63D mutations, which either abolished the phosphorylation site or mimicked

a phosphorylated status. Another serine residue within the SBP-domain, S49, was mutated to alanine in another construct. S49A was used as a negative control since it is also highly conserved but not predicted to be a phosphorylation target (Fig. 3-12).

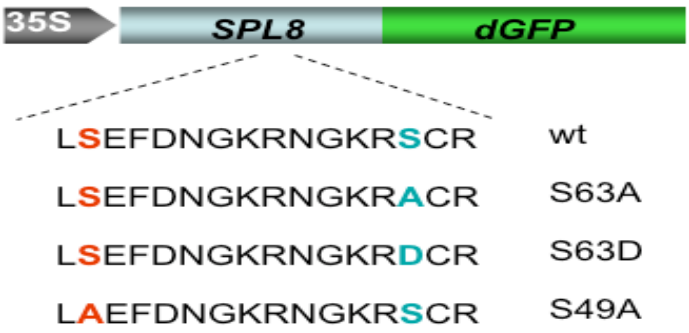

Figure 3-12. Constructs for SPL8 subcellular localization assays. Wild-type and mutated full length SPL8 were fused at C-terminus to double GFP. The three SPL8 mutants have S63A, S63D and S49A mutations, respectively.

3.2.3 Transient expression in tobacco protoplasts

Constructs expressing SPL8 wild-type, SPL8 (S63A), SPL8 (S63D), and SPL8 (S49A), were transformed into tobacco BY-2 protoplasts for a transient expression assay according to a procedure described by Jach et al. (2001).

Fluorescence of GFP expressed as a non-fusion protein and acting as the control was equally distributed over both the nucleus and the cytoplasm (Fig. 3-13A). In comparison, the SPL8 wt-GFP fusion displayed a strong nuclear preference (Fig. 3-13B). The introduced amino acid residue replacements had different effects on the subcellular localization of SPL8. The S63A mutation, by which the phosphorylation site was abolished, showed a nuclear localization preference similar to SPL8 wild-type (Fig. 3-13C), whereas S63D, which mimicked a phosphorylated status, has a ubiquitous distribution like observed for the GFP control (Fig. 3-13D). S49A had no obvious effect on the nuclear localization in comparison to wild-type SPL8 although the overall signal was weak in most cases (Fig. 3-13E).

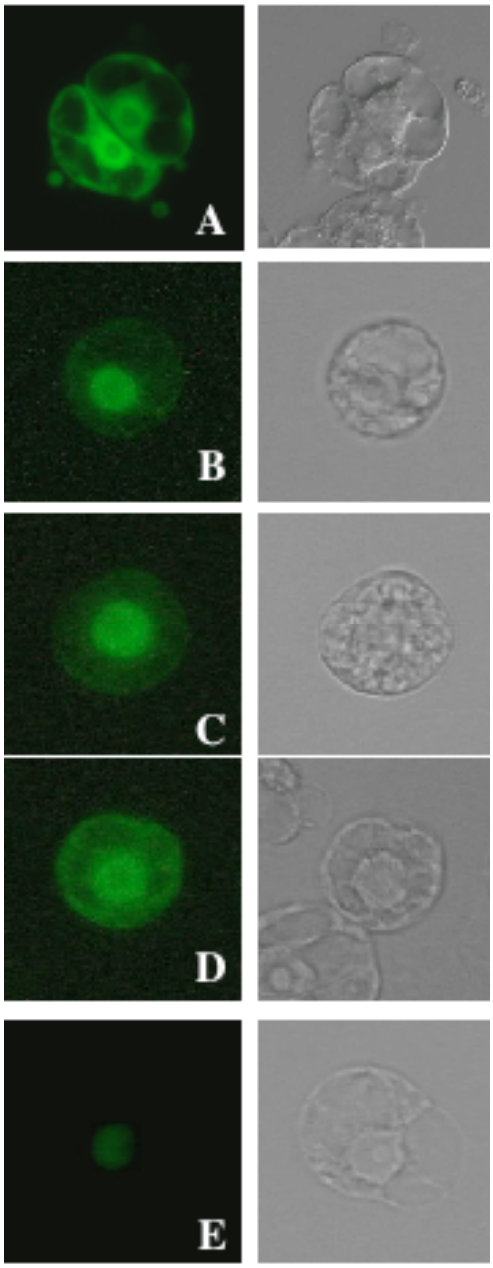

Figure 3-13. Subcellular localization of SPL8-GFP in tobacco protoplasts. Fluorescence microscopy images are shown next to images taken in bright field.

(A) dGFP alone as the control.

(B) SPL8-GFP.

(C) SPL8 (S63A)-GFP.

(D) SPL8 (S63D)-GFP

(E) SPL8 (S49A)-GFP.

The transient expression of SPL8-GFP fusions supports the hypothesis that SPL8 is a nuclear protein and that its nuclear localization is subject to the phosphorylation status of S63 within the SBP-domain. But tobacco protoplasts represent a heterologous environment and there remained a possibility that some key elements involved in SPL8 localization were absent. The outcome of the transient expression assay might thus not reflect the behavior of SPL8 under native conditions.

3.2.4 Stable expression *in Planta*

Therefore, the four constructs were also introduced into Arabidopsis plants for stable transgenic expression. Over 100 independent transgenic lines were isolated per construct and examined for GFP-fluorescence in the above earth tissues. Unexpectedly, only one to three transgenic lines with detectable GFP-fluorescence could be obtained for each of the mutated SPL8-GFP fusion constructs. No transgenic plants were detected to express wild-type SPL8-GFP fusion protein.

GFP alone has been shown to have a ubiquitous expression in plant cells (e.g. Ishida et al., 2004), therefore was not incorporated as a control. As shown in Fig. 3-14, the three mutated SPL8-GFP fusions displayed similar localization patterns as in the transient assay using tobacco protoplasts. Without the phosphorylation-potential as in the case of the S63A mutation, SPL8-GFP fusion proteins are mainly located in the nucleus (Fig. 3-14A). SPL8 (S63D), the mutant simulating a phosphorylated status, shows a more equal distribution over nucleus and cytoplasm (Fig. 3-14B). Mutation of S49, a conserved residue not predicted to be prone to phosphorylation, did not change the property of SPL8 being preferentially nuclear localized (Fig. 3-14C).

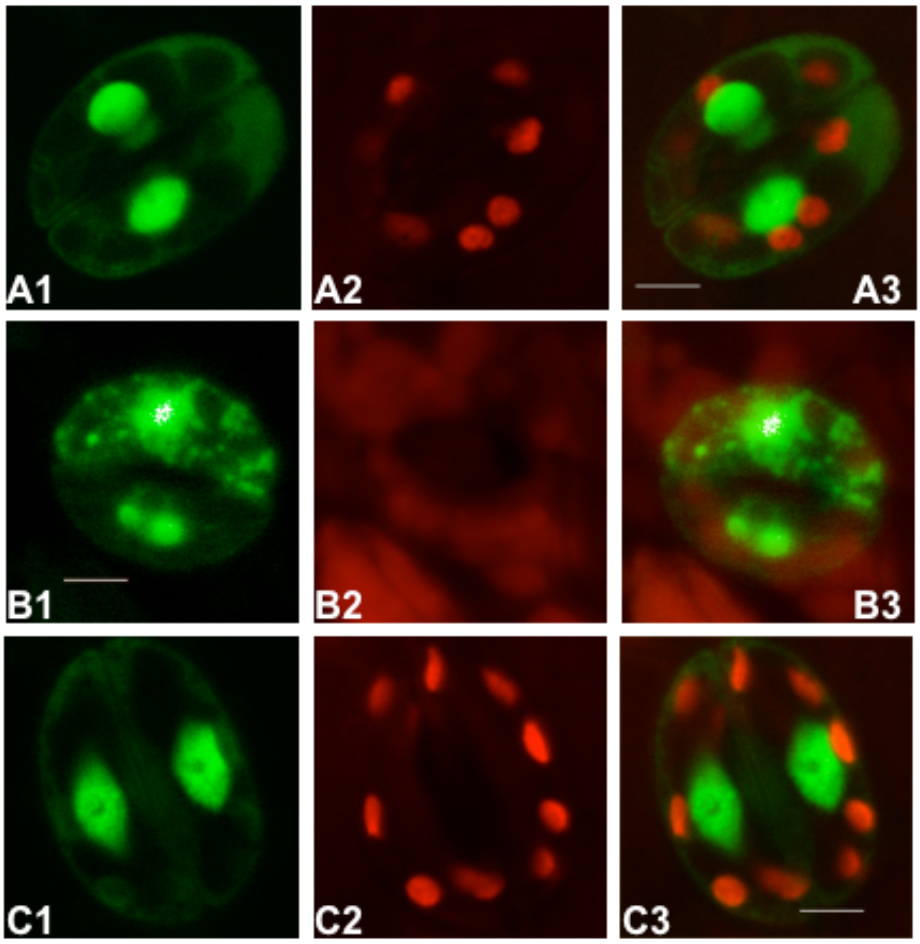

Figure 3-14. Subcellular localization of SPL8s *in Planta* by confocal laser-scanning microscopy imaging. 1, GFP; 2, chloroplast auto-fluorescence; 3, 1 and 2 merged images. Bar = 5 □m.

(A) SPL8 (S63A)-GFP.

(B) SPL8 (S63D)-GFP.

(C) SPL8 (S49A)-GFP.

3.2.5 Discussion

SBP-domain proteins are likely transcription factors capable of sequence-specific DNA-binding and believed to be targeted to the nucleus due to the presence of a conserved bipartite NLS. For the maize SBP-domain protein LIGULELESS1, its nuclear localization had been shown by Moreno et al. (1997). Both the transient and stable expression studies described here,

also strongly support that SPL8 represents a nuclear targeted protein, an essential feature for a putative transcriptional regulator of nuclear genes.

The conserved NLS of SPL8 and other SBP-domain proteins locates within the SBP-domain, where it overlaps with residues responsible for the formation of zinc-dependent DNA-binding function. A conserved serine residue, predicted to be a target for phosphorylation, can be found within the NLS. That the residue can influence the function of the NLS through post-transcriptional modifications may find indirect experimental support through the observations made on the subcellular distribution of SPL8 after replacement of the respective serine. The results obtained here suggest that SPL8 would be targeted to the nucleus when the status of S63 is dephosphorylated. A phosphorylated S63 probably means that the SPL8 protein is redirected to or retained in the cytoplasm. Phosphorylation is one of the most frequent post-transcriptional modifications to regulate protein function and plays a role in many signaling cascades. The regulation of nuclear localization by phosphorylation of SPL8 and other SBP-domain proteins provides a topic for further studies directed towards the elucidation of the signals regulating the post-transcriptional modifications.

3.3 Identification of SPL8 interacting proteins

The first section of the result part showed that SPL8 acts as a local regulator in a subset of GA-mediated developmental aspects revealed by the characterization of loss- and gain-of-function mutants. In the second section, it was shown that SPL8 is a nuclear protein and experimental evidence suggested that its intracellular distribution may be regulated by its phosphorylation status. The results obtained so far strongly supports the initial idea that SPL8 acts as a transcription factor. It is well established that protein-protein interactions are essential in the regulation of gene expression by transcription factors. Additional efforts were therefore made to identify proteins interacting with SPL8 in order to integrate SPL8 further into a regulatory network.

3.3.1 The GAL4 yeast two-hybrid system

Recently, many biochemical, physical, and genetic techniques have been developed to detect specific protein-protein interactions, including yeast two-hybrid (Y2H), co-immunoprecipitation, fluorescence resonance energy transfer (FRET) and all were applied successfully in diverse cases (Immink et al., 2002; Yang et al., 2001; Pelaz et al., 2001). Of these different approaches yeast two-hybrid screening seems more suitable than other techniques for identifying SPL8 interactors mainly for its unique features. First, it is more sensitive than most of the other techniques and capable of detecting very weak and transient protein interactions, which might still be biologically meaningful. Second, the Y2H assay is performed *in vivo* providing good conditions for detecting interactions between proteins expressed in their native conformations. Third, it also can be used for detecting functionally critical residues in protein-protein interaction and pinpoint the interaction domains.

In the Y2H system, the gene of interest is fused to the coding region of a yeast transcription factor binding domain (BD), which then will be used as a "bait" to screen an expression library of "preys", i.e. proteins fused to a transcription factor activation domain (AD). When bait and prey interact, the DNA-BD and AD are brought into proximity and become capable of activating the transcription of selected reporter genes (Fig. 3-15A). The expression of

these reporter genes provide yeast the capacity to grow on particular nutrient-deficient media.

The MATCHMAKER GAL4 Two-Hybrid System 3 (Clontech) was used in this study. This system consists of two vectors and two yeast strains. A bait gene is expressed as a fusion to the GAL4 BD in vector pGBKT7, which gives the transformed yeast strains the capacity to grow on Trp-deficient medium. cDNA libraries are expressed as fusions to the GAL4 AD in vector pGADT7, which allows the transformed yeast strains to grow on Leu-deficient medium. Two yeast strains, Y187 and AH109 were used as hosts for the expression of the bait and cDNA library constructs. Since they have different mating types, two hybrid screening can be done by yeast mating.

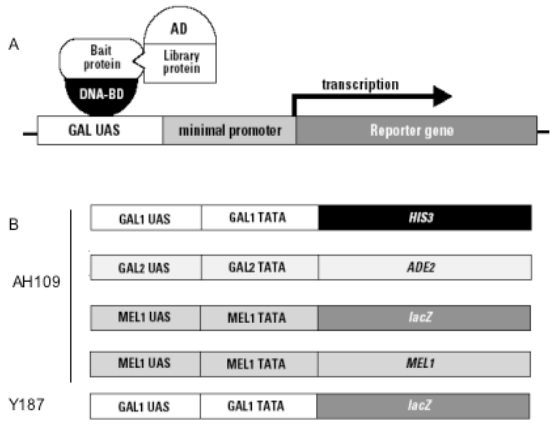

Figure 3-15. The MATCHMAKER3 GAL4 yeast two-hybrid system.

(A) Illustration of the basic mechanism of Y2H.

(B) Reporter constructs in the two host strains used in this system.

As shown in Fig. 3-15B, Y187 contains the lacZ reporter gene under control of the *GAL1* upstream activating sequences (UAS). While AH109, the yeast strain for cDNA libraries, contains four reporters, *ADE2*, *HIS3*, *MEL1*, and *lacZ*, under the control of *GAL4* UASs and TATA boxes. The *ADE2* reporter alone provides strong nutritional selection. The option of using *HIS3* selection reduces the incidence of false positives and allows control over the stringency of selection. Furthermore, *MEL1* and *lacZ*, which encode a-galactosidase and b-galactosidase, respectively, allow the application of blue/white screening on X-a-Gal/X-Gal indicator plates. The four reporter genes used in AH109 thus virtually eliminated false positives.

3.3.2 Construction and characterization of SPL8 baits

3.3.2.1 SPL8 baits

To identify proteins capable of interacting with SPL8, a construct expressing full length SPL8 fused at its N-terminus to the GAL4 BD was generated. In addition, constructs were generated to express truncated versions of SPL8 fused at their N-termini to the GAL4 BD. These should allow a functional dissection of the SPL8 protein with help of the Y2H system.

As mentioned in the introduction part, the sequences of SBP-domain proteins generally display a high degree of heterogeneity outside the SBP-domain. Still, some conserved motifs exist within different subfamilies, for instance, the previously mentioned ankyrin repeats shared by the largest SBP-domain proteins.

By sequence comparison, SPL8-like proteins contain a conserved sequence upstream of the SBP-domain consisting of the core motif, 'RIGLNL' (Fig. 3-16). A comparable motif could not be detected in other protein sequences available in the electronic databases and its function therefore, remained unknown. Because of the possibility that this conserved motif represents an activation domain, several truncated SPL8 baits were constructed to test the capacity of RIGLNL motif to activate transcription in the Y2H system. Furthermore, it has been suggested that phosphorylation regulated by phosphatases and/or kinases may play an important role in SPL8 subcellular localization. Therefore, SPL8 mutants with an altered S63, the putative phosphorylation site, were also generated as baits as they could become useful for confirming interactions between SPL8 and kinases or phosphatases.

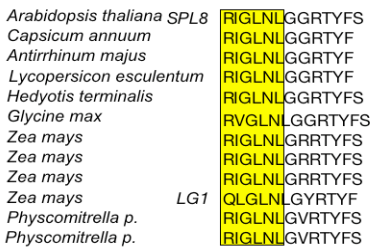

Figure 3-16. Alignment of several SPL8-like proteins showing the conserved RIGLNL motif. Courtesy M. Riese.

3.3.2.2 Self-activation assay

Although Y2H systems are sensitive and versatile, they have some potential limitations. The most frequently encountered problem is that some bait proteins such as transcriptional activators have intrinsic transcriptional activating domains, leading to strong background. Before the start of the Y2H screening of expression libraries, a self-activation assay was performed to reduce the possibility that too many false positives would be obtained.

Leaky HIS3 expression is usually the cause of false positives and has to be inhibited with the His3p competitive inhibitor, 3AT, to suppress background growth on YSD-His plates. The 3AT concentration has to be optimized because a high concentration in the medium can kill freshly transformed cells.

SPL8 baits, together with an empty BD vector as the negative control, were independently transformed into strain AH109 and grown on YSD-Trp plates. Individual colonies were inoculated on different nutrient-deficient media supplemented with a concentration series of 3AT. Colony development was recorded after one week.

	-W	**-WH + 3AT***					**-WA**	**-WAH**
		0 mM	4 mM	6 mM	8 mM	12 mM		
pGBKT7	+	+/-	-	-	-	-	+/-	+/-
ZY115	+	+/-	-	-	-	-	+/-	+/-
ZY113	+	+	-	-	-	-	+/-	+/-
ZY114	+	+	+	+	+	+	+	+
ZY116	+	+	+	+	+	+	+/-	+/-
ZY118	+	+/-	-	-	-	-	+/-	+/-
ZY119	+	+	-	-	-	-	+/-	+/-
ZY120	+	+/-	-	-	-	-	+/-	+/-

Table 3-2. Self-activation assay. +, strong growth; +/-, weak growth, -, no growth. -W, media minus Trp; -H, minus His; -A, minus Ade. *The amount (mM) of 3AT added indicated in the row below.

As shown in Table 3-2, SPL8 full length protein (ZY115) resulted in slight background growth on -Trp/-His and -Trp/-His/-Ade plates but this could

be suppressed by addition of 4 mM 3AT to the media. SPL8 (S63A) and SPL8 (S63D) displayed similar self-activation patterns, indicating that S63 does not affect the trans-activating capacity of SPL8 in yeast. The N-terminal truncated SPL8 (ZY113), which contains sequences including and downstream of SBP-domain (Fig. 3-17), also did not show much self-activation.

In contrast, two other SPL8 truncations displayed a high self-activating capacity as deduced from the strong growth on -Trp/-His/-Ade plates. ZY114 expresses a truncated SPL8 containing SPL8 sequence upstream of the SBP-domain (Fig. 3-17). The other strongly activating construct pZY116 contains N-terminal truncation but preserved RIGLNL motif (Fig. 3-17). These results suggest that the novel RIGLNL motif might be responsible for the trans-activating capacity of SPL8 in yeast. The only discrepancy seems to be that ZY118, the construct expressing RIGLNL alone (Fig. 3-17), did not show background growth. A possible explanation is that this severely truncated version of SPL8 can not fold properly, resulting in its incapacity to activate.

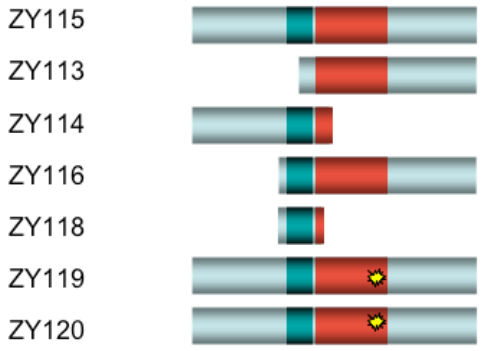

Figure 3-17. Sequence alignment of SPL8 baits constructed for yeast system. Red bar represents SBP-domain; green bar, RIGLN motif, yellow star, mutated site at S63.

3.3.3 Y2H library screening

3.3.3.1 Arabidopsis cDNA libraries

This study used two Arabidopsis cDNA libraries pre-transformed in AH109 (kindly provided by H. Sommer, MPIZ). cDNAs were reverse-transcripted from Arabidopsis total RNAs extracted from whole plants and from shoot apex before floral induction (Davies et al., 1996). Two different

priming methods were used in making cDNA libraries to combine their advantages. Oligo(dT) priming ensures that full length clones can be obtained and 3' ends will be well-represented in the library. Random priming generates a wider size-range of cDNAs, which are useful in functional dissection. Using two ways to prime is beneficial in representing all portions of the gene.

3.3.3.2 Yeast mating for library screening

The yeast mating method takes advantage of the fact that haploids of opposite mating types can mate to form diploid cells and hence presents a convenient method of introducing two different plasmids into the same yeast cell (Finley and Brent, 1994). Compared with the transformation method, yeast mating has several advantages.

First, the transformation method is problematic when yeast cells do not have healthy growth as a result of disturbed cellular activities by the bait. Yeast mating is more suitable under such conditions. Second, diploid yeast cells are more vigorous than haploid yeast cells, hence yeast mating allows the detection of weak and rare protein-protein interactions. Finally, the reporters are less sensitive to transcription activation in diploids than in haploids thus yeast mating will give less background growth by baits that weakly auto-activate.

3.3.4 Results from the Y2H screening

Wild-type full-length SPL8 transformed in Y187 was used as the bait to screen two cDNA libraries pre-transformed in AH109. The mating mixture was plated on both medium- and high-stringent selective media. In total, there were around 100 colonies growing on –WLHA + 4mM 3AT plates. PCRs were performed on DNA extracted from these colonies using a pGADT7-specific primer pair to amplify the corresponding prey coding DNA. Sequences were "BLASTed" against the GenBank database and annotated according to the MIPS and TIGR Arabidopsis sequence databases (see Material and Methods). Final results are listed in Table 3-3, with the identified genes classified according to their functional categories.

Most proteins identified from the library screening appear to be biologically less relevant to come to a better understanding of SPL8 function.

Such proteins include those facilitating protein folding and degradation, participating in the metabolism of lipid and protein, being targeted to other organelles such as chloroplast and mitochondria. These proteins were excluded for further analysis as were proteins without known function or with no functional domains assigned.

Among the identified putative interactors of particular interest are those that can be grouped in either of the following two classes, those involved in transcriptional regulation and those in phosphorylation. Six putative transcriptional regulators were identified from the Y2H screening, including the basic- Helix-Loop-Helix proteins bHLH046 (At5g08130) and bHLH6 (At1g32640), the recombination signal sequence-recognition protein (At3g28730), the trihelix DNA-binding protein (At1g33240), and a histone deacetylase (HD2B, At5g22650). The expression files of their corresponding genes were analyzed with the Genevestigator, an internet-based microarray database (https://www.genevestigator.ethz.ch/; Zimmermann et al., 2004). Unfortunately, all of these genes showed a constitutive expression pattern with low occurrence in stamen, making them therefore unlikely candidates for important SPL8 interactors.

The second class of putatively interesting candidates consists of those involved in protein phophorylation, i.e. kinases and phosphatases. As suggested by SPL8 subcellular localization assays, phosphorylation could play an important role in the functionality of SPL8. Several genes encoding kinases and phosphatases appeared as putative interactors of SPL8 in the Y2H screen, and including genes encoding a calcineurin-like phosphoesterase (At3g09970), an SNF1-related protein kinase (At3g01090), a ser/thr protein phosphatase (At3g19980), a phosphatase-related protein (At1g13750), a mitogen-activated protein kinase kinase (At4g26070), and a phosphatase-related protein (At4g23570). Their expression profiles were also analyzed with Genevestigator (Zimmermann et al., 2004). These and other identified genes were found to be constitutively expressed, and relatively low in stamen, seedling and shoot apex with one exception, At3g09970, in the following referred to as CAP (for Calcineurin-like Phosphoesterase), which has a stamen-enriched expression pattern. Based on its annotated function and expression pattern, CAP was selected for further analysis.

Table 3-3. Proteins identified from Y2H

AG code	Annotation (Occurance)	Available mutants
TRANSCRIPTIONAL REGULATION		
At5g08130	bHLH046 (2x)	SALK_044682
At1g32640	bHLH6 (1x)	GABI-KAT 293F05
At3g28730	Recombination signal sequence recognition protein, putative (1x)	GABI-KAT307C08
At1g33240	trihelix DBP, putative (1x)	SALK_044308
At5g22650	histone deacetylase (HD2B) (2x)	
PHOSPHORYLATION		
At3g09970	calcineurin-like phosphoesterase (21x)	GABIKAT 246A02
At3g01090	SNF1-related protein kinase (3x)	
At3g19980	ser/thr protein phosphatase (1x)	SALK_122405
At4g26070	MEK1 (mitogen-activated protein kinase kinase, MAKK) (1x)	SALK_015914
At1g13750	phosphatase-related (2x)	SALK_019558
At4g23570	phosphatase-related (1x)	SALK_026971
PROTEIN FOLDING AND DEGRADATION		
At4g34960	Cyclophilin (2x)	
At5g02500	DnaK-type molecular chaperone (1x)	
At1g10350	heat shock protein (1x)	
At5g28540	luminal binding protein (2x)	
At2g16600	cytosolic cyclophilin (1x)	
At5g61790	calnexin-like protein (1x)	
At5g42020	luminal binding protein (1x)	SALK_047956
At3g14240	subsilinase (4x)	
At1g20160	subsilinase (2x)	
At3g25230	rotamase FKBP(ROF1) (1x)	

Table 3-3. Preys identified from Y2H (continued)

AG code	Annotation (Occurance)	Available mutants
METABOLISM		
At2g04570	putative GDSL-motif lipase/hydrolase (12x)	
At1g20620	catalase-3 (3x)	
At1g47128	cysteine proteinase RD1A (14)	
At3g10860	ubiquinone-binding protein (17x)	
At1g23190	phosphoglucomutase (3x)	
TARGETTED TO OTHER ORGANELLES		
At1g80380	chloroplast protein (8x)	
At2g22360	DnaJ-like protein, chloroplast protein (4x)	
At3g61050	CalB, mitochondria protein (2x)	
At5g20250	seed imbitition protein-like, chloroplast protein (1x)	
METAL-RELATED PROTEINS		
At2g46800	Zinc transporter, putative (1x)	
At5g18650	C2HC4-type RING finger protein (1x)	
At5g50740	copper binding (1x)	
At3g15353	copper binding (2x)	
FUNCTION UNKNOWN AND MISCELLANEOUS		
At5g24620	unknown protein (5x)	
At4g24690	unknown protein (15x)	
At4g14160	unknown protein (5x)	
At3g07195	unknown protein (8x)	
At3g05670	unknown protein (2x)	
At5g54510	DFL1 (2x)	SALK_013458
At3g44400	disearse resistance protein (TIR-NBS-LRR class) (1x)	
At3g07940	GTPase-activating protein (1x)	
At1g09070	C2-domain-containing protein (1x)	
At2g20990	C2-domain-containing protein (4x)	
At5g47710	C2-domain-containing protein (2x)	

In addition to candidate interactors putatively involved in transcriptional regulation and phosphorylation, hormone-related factors are also interesting. As discussed in the first section of the results, SPL8 is involved in GA-mediated developmental processes. Although no GA-related genes were identified from this screening, an auxin-responsive gene, *DFL1* (*DWARF IN LIGHT 1*, Nakazawa et al., 2001), appeared.

DFL1 is a GH3 protein that belongs to the Arabidopsis GH3 family. GH3 genes were among the earliest auxin-responsive genes, whose transcript levels can increase many folds in response to auxin (Nakazawa et al., 2001). One experiment demonstrated that *DFL1* was upregulated 33 times after auxin treatment (Zhao, et. al., 2003). According to Genevestigator, *DFL1* is most highly expressed in the shoot apex and seedlings in comparison to other tissues and stages. The dominant mutant *dfl1-D* displayed a pleiotropic phenotype including a short hypocotyl and stem, small leaves, and inhibited later root growth (Nakazawa et al., 2001). These pleiotropic effects to some extend correlate to several phenotypical aspects of the *SPL8* gain-of-function transgenic plants described above. Additional support for a possible meaningful interaction between DFL1, GA and SPL8 can be found in a recent report demonstrating that auxin regulates root elongation by enhancing GA-responsiveness of RGA degradation (Fu et al., 2003). Therefore, *DFL1* was taken as a candidate for further analysis.

3.3.5 Reverse genetics of putative interactors

CAP (At3g09970) has been annotated by TIGR (The Institute for Genomic Research; www.tigr.org) to serve as a protein serine/threonine phosphatase. CAP is 309 amino acids in length, with the predicted phosphatase activity at its N-terminus. *CAP* has immediately next to it a close homolog At3g09960 (referred to as CAPH, for CAP Homolog), obviously a result of a recent duplication event. A BLAST search revealed one rice gene (9632.m03207) that shares a high sequence similarity to *CAP*, and will be referred to as *OsCAPH*, for *Oryza sativa* CAP Homolog (Fig. 3-18A). Besides these two highly related genes, there were no other obvious homologs found for *CAP* in the databases. A T-DNA insertion mutant could be obtained for

CAP from GABI-KAT (German Plant Genomics Research Program; www.gabi-kat.de). The T-DNA was found to be inserted into the third exon of *CAP* (Fig. 3-18B), resulting in a null mutation as could be proved by RT-PCR with a CAP-specific primer pair. Respective homozygous mutants were grown under LD and SD conditions, but no alteration in fertility or other obvious phenotypic alterations were observed under both conditions.

DFL1 (At5g54510) belongs to the Arabidopsis GH3 family, which includes several other genes like At4g37390, At2g23170, At1g59500, At4g27260, and At1g28130 that all display a high degree of sequence similarity. A T-DNA mutant line SALK_013458, was obtained from ABRC (Arabidopsis Biological Resource Center; www.arabidopsis.org/abrc/), but no mutant phenotype observed under the LD and SD growing conditions tested.

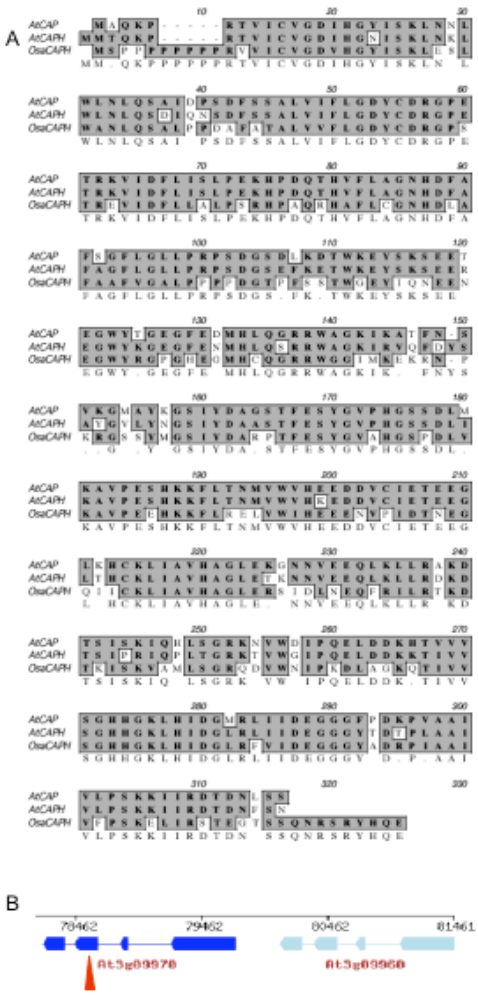

Figure 3-18. (A) Sequence alignment of *CAP* with its Arabidopsis homolog *CAPH* and

presumed rice ortholog *OsCAPH*. (B) T-DNA insertion in *CAP* as found in the GABI-KAT 246A02 mutant line. Arrow indicates the T-DNA insertion site.

3.3.6 Discussion

A Y2H screening with full-length wild-type SPL8 was performed to identify SPL8-interacting proteins. Over one hundred positive colonies appeared from two library screenings representing dozens of proteins as determined by subsequent sequence analysis. Database-based sequence comparisons predict the major part of these genes to be involved in some general cellular activities, such as proper folding of proteins and the metabolism of lipids and proteins. The interactions between SPL8 and these proteins might be real under native conditions, i.e. actually happening *in planta*. Not only might this be difficult to prove, but, more importantly, also no obvious relevant information helping to uncover to role of SPL8 in development can be obtained. Therefore, among the putative interactors, transcription regulators and kinases/phosphatases were considered to be of more biological relevance with respect to the properties of SPL8.

Expression profiles represent another important criterion in the selection of putative candidates. Genes are generally expected to have similar, or at least significantly overlapping, expression profiles, necessary for their products to be able to interact. Database queries with Genevestigator (Zimmermann et al., 2004) revealed a few genes with specific expression patterns overlapping that of SPL8.

The first one *CAP*, encoding a protein with ser/thr protein phosphatase activity, is found to be highly expressed in stamen. Its function could be of importance with respect to the predicted post-transcriptional modification of a conserved serine within the SBP-domain and likely to be critical for the subcellular distribution of SPL8 as described in the previous result section. Unfortunately, no detectable mutant phenotype could be observed for *CAP*. This might be explained by assuming functional redundancy due to the presence of the highly identical *CAPH*. However, the expression of *CAPH* is found to be ten fold lower than that of CAP, especially in stamen. Therefore, it

may not be useful in generating double mutant of these two genes.

The other gene, *DFL1*, one of the GH3 genes in Arabidopsis, was selected because of its responsiveness to the phytohormone auxin. It was recently found that DFL1, as well as several its homologous GH3 proteins, displays an enzymatic activity in the conjugation of IAA to amino acids (Staswick et al., 2005). From this perspective the meaning of the DFL1-SPL8 interaction seems difficult to explain.

There may be several explanations for the rather unsatisfying results of the Y2H screening. One of these might be that expression of SPL8 is highly restricted temporally and spatially (Unte et al., 2003). Therefore, proteins interacting with SPL8 might be expressed also in a spatiotemporal way, leading to the underepresentation of the encoding genes in the two Arabidopsis cDNA libraries. Consequently, the chance to pick up transcripts representing true SPL8 interactors may be low. For a successful Y2H screening, it might be necessary to generate cDNA libraries from developing floral buds when mRNAs expressed in early stages of anther are better represented in total mRNAs extracted. Another reason might be resulted from the detrimental effect of SPL8 expression on yeast cells. The expression of full-length wild-type SPL8 somehow disturbed the normal celllular activity of yeast cells, dramatically reducing yeast growth rate compared with empty BD alone, thereby hindered the sensitivity of detecting weak interactions. Accordingly, proteins weakly or transiently interacting with SPL8 may no be identifiable by this approach.

4. General Discussion

SBP-box genes encode plant-specific transcription factors. Among the 16 Arabidopsis SBP-box genes, *SPL8* represents one of few whose functional role has been brought into light (Unte et al., 2003). *SPL8* was known to be involved in reproductive growth as deduced from its loss-of-function phenotype, but it was not clear how it implements such a function. Therefore, it would be enlightening to integrate *SPL8* in an existing regulatory network controlling reproductive development.

The experimental results presented in this thesis provide evidence that the role of SPL8 might be through GA-mediated regulatory network, hence give a more detailed insight in the role of *SPL8* in Arabidopsis development.

4.1 *SPL8* is involved in GA-mediated anther development

The most prominent function of *SPL8* involves Arabidopsis anther development. *SPL8* loss- and gain-of-function both display male sterility, but as a result of different mechanisms. In the absence of a functional *SPL8*, anther developmental defects start at an early stage when local divisions in the L2 cell layer give rise to four archesporial cells in the wild-type anther. Consequently, the normal four-lobed anther structure is perturbed in *spl8* mutants (Unte et al., 2003). It suggests that *SPL8* is responsible for receiving an unknown signal for anther cell patterning.

On the other hand, constitutive *SPL8* over-expression has no effects at early stages of anther development. Only at anther stage 13, the normal dehiscence program becomes disturbed by the constitutive and ectopic *SPL8* expression, which leads to male sterility. According to the results of *in situ* hybridization, the expression of *SPL8* in the wild-type anther is temporarily controlled such that it is only transcriptional active before stage 7 (Unte et al., 2003). Taken together, the observations strongly suggest that the activity of

SPL8 is only required within a specific developmental window of time for proper anther development.

Owing to the agricultural and environmental importance of anther development, extensive studies have been conducted. As a result, many factors are found to act on this process. To get a better understanding of the functional mechanism of *SPL8*, it is necessary to put *SPL8* into the context of the regulatory network controlling anther development.

Phenotypical comparisons hinted that *SPL8* might be involved in GA-mediated anther development. Gas play major and diverse roles in anther development. Whereas a GA-deficiency or -insensitivity typically results in post-or pre-meiotic defects and shortened stamen filaments (Cheng et al., 2004; Izhaki et al., 2002; Jacobsen and Olszewski, 1991), a GA-overdose or constitutive GA-response causes male sterility due to anther non-dehiscence (Colombo and Favret, 1996; Murray et al., 2003). These anther developmental defects correlate well with those shown by *SPL8* loss- and gain-of-function mutants respectively as demonstrated by comparative histology.

Although the positive regulatory role of SPL8 in GA-mediated anther development is supported by most experimental data, there still remain some discrepancies. The GA-deficient mutant *ga1-3* has normal anther development until the formation of tetrads after which pollen of *ga1-3* does not release from tetrads, leading to male sterility (Cheng et al., 2004). In comparison, anther development of *spl8* mutants is defective at a very early stage when the archespores are initiated (Unte et al., 2003). Furthermore, ovule development has not been implicated as controlled by GAs, whereas megaspores of *spl8* mutants are found to degenerate at flower stage 11-12 (defined by Smyth et al., 1990). One possible explanation is that there are other input signals acting on *SPL8* functionality. It would be interesting to find out the genetic interactions of *SPL8* with genes whose mutations affect a similar early stage of anther development. However, no other mutants

disclosed up to now show defects at the initiation of archesporial cells so the unknown factor remains to be determined.

4.2 *SPL8* affects GA-mediated seed germination and root elongation

GAs are necessary for seed germination of wild-type Arabidopsis where they act mainly in counteracting the inhibitory effect of the DELLA protein RGL2 (Lee et al., 2002; Tyler et al., 2004). The GA-deficient mutant *ga1-3* can not germinate without exogenously applied GAs (Koornneef and Van der Veen, 1980), but its germination failure can be rescued by introducing an *rgl2* mutation (Tyler et al., 2004). In addition, *rgl2* as well as the constitutive GA-response mutant *spy*, display PAC-resistant seed germination, suggesting no further requirement for GAs for this process (Tyler et al., 2004; Jacobsen and Olszewski, 1993). The role of GAs on root elongation has recently been uncovered (Fu and Harberd, 2003). Primary roots of *ga1-3* are shorter than those of wild-type, a defect compensated by GA treatment or the removal of both the functions of *RGA* and *GAI* (Fu and Harberd, 2003).

Besides its expression during reproductive growth, *SPL8* transcription is active in seedling stage, albeit not very strong. Not surprisingly, *SPL8* gain- and loss-of-functions display phenotypical alterations during this stage. Constitutive *SPL8* expression reduces the rate of seed germination dramatically and it can not be rescued by exogenously applied GAs. On the contrary, the seed germination of *spl8* mutants displays a higher resistance to the GA-biosynthetic inhibitor PAC than wild-type. Root elongation is affected in the same way such that *35S::SPL8* plants have shorter primary roots and *spl8* mutants have longer ones as compared to those of wild-type. These results support a negative role for *SPL8* during the seedling stage in a GA-dependent way.

Further evidence for this hypothesis comes from RT-PCR data. The GA-signaling response is enhanced upon the removal of negative regulators. Subsequently, GA-biosynthetic genes like *GA5* will be downregulated due to

a feedback control mechanism. On the other hand, constitutive expression of negative regulators will reduce the GA-signaling response, and GA-biosynthetic genes will consequently become upregulated in compensation. These changes can be detected by the transcript levels of related genes, like *RGL2* and *GA5*. Indeed, *RGL2* and *GA5* levels are found to be decreased in *35S::SPL8* plants, demonstrating an increased endogenous GA level due to the constitutive expression of a negative regulator. A discrepancy regarding to the *GA5* level in the *spl8* loss-of-function mutant is probably due to a disturbed feedback control as discussed in the respective result section.

4.3 *SPL8* is not under the direct transcriptional regulation of GAs

GA regulation is implemented in plant development as well as its own biosynthesis partly through transcriptional regulation. For example, GAs regulate the initiation of trichomes by transcriptionally upregulating the expression of *GLABROUS1*, which encodes a positive regulator in GA-signaling response (Perazza et al., 1998). Genes encoding for negative regulators, like *RGL2*, are downregulated in a GA-dependent way (Bassel et al., 2004). Bioactive GAs negatively regulate the abundance of their biosynthetic genes *GA4* and *GA5* in a dose-dependent manner (Chiang et al., 1995; Cowling et al., 1998; Xu et al., 1995; 1999). To the contrary, genes encoding enzymes involved in GA catabolism such as *AtGA2ox6* are upregulated by bioactive GAs (Wang et al., 2004).

A 5 fold induction of *SPL8* transcript level was revealed by microarray-mediated expression profiling in *ga1-3* apex 24 hr after treatment with GAs (M. Schmid and D. Weigel, personal communication of unpublished data and referred to in Unte et al., 2003). This posed an interesting question: is *SPL8* under direct transcriptional control of GAs? The answer is probably negative. No obvious differences in *SPL8* levels could be obtained after wild-type apices were treated with bioactive GAs for 2hr, a period when GA-biosynthetic genes are downregulated due to the feedback mechanism. The 24 hr between GA application and first isolation of mRNA as in the above-mentioned microarray experiment represents an unusually long period for

examining immediate phytohormone effects. Although *SPL8* displays a lower expression in inflorescence of *ga1* mutant compared to that of wild-type, it is unlikely that *SPL8* is under the direct transcriptional control of GAs.

4.4 Is the nuclear localization of SPL8 GA-dependent?

As expected for a transcription factor and to fulfill its regulatory function, SPL8 has to localize to the nucleus. Interestingly, its intracellular localization is predicted to be affected by phosphorylation. Experimental data revealed that the abolishment of possible phosphorylation through mutation of a particular conserved serine residue in the SBP-domain causes SPL8 protein to accumulate preferentially into nucleus. To the opposite, a Ser to Asp mutation, which resembles a phosphorylated status, seems to retain the protein into cytoplasm. Considering the possible effect of phosphorylation and the involvement of *SPL8* in a GA-signal response, it is tempting to propose GAs as the candidate for regulating such a modification.

It is known that GAs regulate its signaling components not only at the transcript level but also at the post-transcriptional level. A well-studied mechanism is the GA-dependent proteasome-mediated protein degradation. As mentioned before, GA-mediated plant development is repressed by DELLA proteins in the absence of GAs (Peng et al., 1999). The inhibitory effects of these repressors are counteracted by GAs through proteasome-mediated protein degradation (Fu et al., 2004; Dill et al., 2004). GAs cause Arabidopsis RGA and its functional orthologs in barley and rice to disappear from the nucleus (Fu et al., 2004; Dill et al., 2004; Itoh et al., 2002; Sasaki et al., 2003). This re-localization can be inhibited by specific inhibitors of ser/thr protein phosphatases or of the 26S proteasome (Itoh et al., 2002; Sasaki et al., 2003), implying that GAs induce the phosphorylation of DELLA proteins, which will then become degraded in a proteasome-mediated manner.

GA-regulated phosphorylation also plays a role in the intracellular localization of transcriptional regulators. REPRESSION OF SHOOT GROWTH (RSG) is a tobacco (*Nicotiana tabacum*) transcriptional activator with a basic leucine zipper domain that regulates endogenous amounts of

GAs by affecting the expression of *GA3*, a GA-biosynthetic gene (Fukazawa et al., 2000). The intracellular localization of RSG responds to GA treatment such that an increase in GA level results in a cytoplasmic relocalization and a reduced GA level again favors the nuclear localization of RSG (Ishida et al., 2004). A particular serine to alanine mutation abolished this GA-dependent re-localization, suggesting that phosphorylation regulated by GA at the respective serine plays an important role in the subcellular localization of RSG (Ishida et al., 2004).

Similar to RSG, the subcellular localization of SPL8 may be affected by GA-dependent phosphorylation. It remains an open question to be investigated.

4.5 Future perspectives

The studies reported here provide experimental support for a dual role of *SPL8* in a subset of GA-mediated developmental processes, including anther development, seed germination and root elongation.

As suggested by its local effects, *SPL8* is expected to function relatively late within the GA-signaling cascade. There are many GA mutants available in Arabidopsis, including GA-biosynthetic mutants, GA-insensitive mutants and GA-constitutive response mutants, represented by *ga1-3*, *sly1* and *spy-3* respectively. All of those mutants show a male sterile phenotype (Koornneef and Van der Veen, 1980; Steber et al., 1998; Jacobsen and Olszewski, 1993). *SLY1* and *SPY* represent components acting early in the GA-signaling cascade as they regulate a full-spectrum of GA-mediated developmental processes. It would hence be informative to analyze the genetic interactions between *SPL8* and these two genes by creating double mutants. DELLA proteins are the other group of important GA-signaling regulators (for review, see Fleet and Sun, 2005). But due to a high functional redundancy within this group, double mutants are hardly expected to provide useful information.

With the help of modern microarray technology, it is now possible to compare the expression profiles of all Arabidopsis genes among different treatments and genetic backgrounds. *SPL8* loss- and gain-of-function phenotypes display alterations during seedling stage as well as during anther development, likely correlated to altered expression of SPL8 target genes. Therefore, comparative expression profiling among wild-type, *SPL8* loss- and gain-of-function mutants will provide valuable information for the understanding of *SPL8* function in particular and of plant development in general.

5. Summary

Plant development requires a precise and dynamic regulatory network, in which transcription factors play major roles by temporally and spatially regulating gene expression. The SBP-box genes encode a group of plant-specific transcription factors, highly conserved in their DNA-binding domain, but quite heterogeneous outside. Gain- or loss-of-function of several SBP-box genes, including Maize *LIGULELESS1 and Teosinte glume architecture1*, Arabidopsis *SPL3*, *SPL8,* and *SPL14*, display diverse developmental defects, indicating that they function in different developmental processes.

The work presented in this thesis aimed to uncover the role of the Arabidopsis SBP-box gene *SPL8* in plant development and to integrate its function in an existing regulatory network. This has been achieved through the application of molecular genetic, biochemical and microscopic techniques.

As deduced from its semi-sterile loss-of-function phenotype, previous studies have shown that *SPL8* affects reproductive development. Remarkably, and extensively described in this thesis, *SPL8* gain-of-function also caused sterility. However, unlike the loss-of-function mutant in which anther development is defective at early stage of sporogenesis, gain-of-function affects fertility through the non-dehiscence of anthers. Furthermore, constitutive *SPL8* expression resulted in a pleiotropic phenotype partly resembling gibberellin-deficient mutants. Although some phenotypical changes could be rescued by exogenous GAs, seed germination, root elongation and fertility could not. Molecular data indicated that genes involved in GA biosynthesis/response are affected in *SPL8* overexpressing lines, suggesting altered endogenous GA levels.

Because SPL8 is assumed to encode a transcription factor, its nuclear targeting has also been briefly explored using GFP fusion constructs. Within

the SBP-domain, a highly conserved serine residue has been predicted to be a target for phosphorylation. Mutation of this serine residue had a great impact on the subcellular localization of SPL8, suggesting the involvement of post-transcriptional regulation in its function.

To obtain additional information on the regulatory network, SPL8 might be integrated in, a yeast two-hybrid screen was performed. Many putative SPL8 interacting proteins could be identified. The functions of most of these are unknown or predicted to be involved in general cellular activities and therefore not very helpful to clear the function of SPL8. For two putative interactors, selected on the basis of their role in gene regulation and expression pattern, respective mutant plants could be obtained. However, the absence of obvious mutant phenotypes allowed no further conclusions concerning the relevance of their interaction with SPL8.

The experiments in this thesis investigated the role of *SPL8* in plant development. Taken the results together, it could be proposed that SPL8 acts as a local regulator in a subset of GA-mediated developmental processes, positively in anther development, but negatively in seed germination and root elongation. Further studies, aiming at the identification of target genes and genetic interactions with other GA-signaling components, are needed to deepen our understanding of the role *SPL8* plays in these more defined aspects of plant development.

6. ZUSAMMENFASSUNG

Die Entwicklung der Pflanze verlangt ein präzises und dynamisches regulatorisches Netzwerk, in welchem Transkriptionsfaktoren eine bedeutende Rolle bei der zeitlichen und räumlichen Regulation von Genexpression spielen. Die SBP-Box Gene codieren für eine Gruppe pflanzenspezifischer Transkriptionsfaktoren, die eine hochkonservierte DNA-Bindedomäne besitzen, aber nur eine geringe Konservierung außerhalb dieser Domäne. Gewinn- oder Verlustmutationen von verschiedenen SBP-Box Genen, einschließlich *LIGULELESS1* und *Teosinte glume architecture1* in Mais und *SPL3*, *SPL8* und *SPL14* in Arabidopsis, zeigen mannigfaltige Defekte in der Entwicklung. Diese weisen auf eine Rolle der SBP-Box Gene in verschiedenen Entwicklungsprozessen hin.

Das Ziel dieser Arbeit ist, die Rolle des Arabidopsis SBP-Box Gens *SPL8* während der Pflanzenentwicklung aufzudecken und es in die existierenden regulatorischen Netzwerke zu integrieren. Unter Anwendung von molekular genetischen und biochemischen Techniken und der Mikroskopie wurde dieses Ziel erreicht.

Anhand früherer Studien an der *SPL8* Verlustmutante, die einen partiell sterilen Phänotyp zeigt, wurde die Beeinflussung der reproduktiven Entwicklung durch *SPL8* gezeigt. Bemerkenswerterweise, und ausführlich in dieser Arbeit beschrieben, zeigt die *SPL8* Gewinnmutante auch Sterilität. Während in der Verlustmutante die Antherenentwicklung in einem frühen Stadium der Sporogenese gestört ist, beeinflusst die Gewinnmutation die Fertilität durch fehlende Dehiszenz der Antheren. Weiterhin resultiert die konstitutive Expression von *SPL8* in einem pleiotropen Phänotyp, der teilweise die phänotypischen Merkmale von Gibberellin-defizienten Mutanten widerspiegelt. Durch exogene Zugabe von Gibberellinsäure können einige dieser phänotypische Merkmale wiederhergestellt werden, exogene

Gibberellinsäure zeigt aber keinen Effekt auf Samenkeimung, Wurzelstreckung und Fertilität. Molekularanlytische Daten aus der *SPL8* Gewinnmutation weisen auf eine Beteiligung von Gene hin, die auf Gibberellinsäure reagieren und an ihrer Biosynthese beteiligt sind. Dies lässt einen veränderten endogenen Gibberellinsäurespiegel vermuten.

Da SPL8 vermutlich ein Transkriptionsfaktor ist, wurde die nukleäre Lokalisation anhand von GFP Fusionskonstrukten untersucht. In der SBP-Domäne gibt es ein konserviertes Serin, das phosphoryliert werden könnte. Mutationen in diesem Serin wirken sich drastisch auf die subzelluläre Lokalisation von SPL8 aus, was auf eine posttranskriptionelle Regulation hinweist.

Um mehr Information über die regulatorischen Netzwerke, an denen SPL8 beteiligt ist, zu bekommen, wurde ein Hefe-zwei-hybrid Screen durchgeführt. Viele putativ mit SPL8 interagierende Proteine wurden identifiziert. Allerdings sind die Funktionen der meisten unbekannt oder sie spielen eine generelle Rolle in zellulären Aktivitäten. Die identifizierten Proteine konnten daher zur Aufklärung der Funktion von SPL8 nicht beitragen. Anhand ihrer Rolle in der Regulation von Genen und ihres Expressionsmuster wurden zwei putative Interaktoren ausgewählt und die jeweiligen mutanten Pflanzen beschafft. Diese zeigten keinen offensichtlichen Phänotyp und brachten somit keine weiteren Erkenntnisse über die Bedeutung der Interaktion der identifizierten Proteine mit SPL8.

In dieser Arbeit wurde die Rolle von *SPL8* während der Pflanzenentwicklung untersucht. Abschließend kann gesagt werden, dass *SPL8* in ausgewählten Gibberellinsäure-vermittelten Prozessen als lokaler Regulator wirkt. Positiv im Falle der Antherenentwicklung, negative bei der Samenkeimung und Wurzelstreckung. Um unser Verständnis für *SPL8* und dessen Rolle während der Pflanzenentwicklung zu vertiefen, werden weitere Studien zur Identifikation der Zielgene und der genetischen Interaktionen mit weiteren Komponenten des Gibberellinsäure Signalwegs benötigt.

7. LITERATURE CITED

Agashe, B., Prasad, C.K., and Siddiqi, I. (2002) Identification and analysis of *DYAD*: a gene required for meiotic chromosome organisation and female meiotic progression in *Arabidopsis*. Development 129, 3935–3943.

Amagai, M., Ariizumi, T., Endo, M., Hatakeyama, K., Kuwata, C., Shibata, D., Toriyama, K., and Watanabe, M. (2003) Identification of anther-specific genes in a cruciferous model plant, *Arabidopsis thaliana*, by using a combination of *Arabidopsis* microarray and mRNA derived from *Brassica oleracea*. Sex Plant Reprod 15, 213-220.

Archard, P., Vriezen, W.H., Van Der Straeten, D., and Harberd, N.P. (2003) Ethylene regulates Arabidopsis development via the modulation of DELLA protein growth repressor function. Plant Cell 15, 2816-2825.

Azpiroz, R., Wu, Y., LoCascio, J.C., and Feldmann, K.A. (1998) An Arabidopsis brassinosteroid-dependent mutant is blocked in cell elongation. Plant Cell 10, 219-230.

Bai, X.F., Peirson, B.N., Dong, F.G., Xue, C., and Makaroff, C.A. (1999) Isolation and characterization of *SYN1*, a *RAD21*-like gene essential for meiosis in Arabidopsis. Plant Cell 11, 417–430.

Bartel, D.P. (2004) MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 116, 281–297.

Bassel, G.W., Zielinska, E., Mullen, R.T. and Bewley, J.D. (2004) Down-regulation of DELLA genes is not essential for germination of tomato, soybean, and Arabidopsis seeds. Plant Physiol 136, 2782-2789.

Becraft, P.W., Bongard-Pierce, D.K., Sylvester, A.W., Poethig, R.S., and Freeling, M. (1990) The Liguleless-1 gene acts tissue specifically in maize leaf development. Dev Biol 141, 220–232.

Bhatt, A.M., Lister, C., Page, T., Fransz, P., Findlay, K., Gareth, H.J., Dickinson, H.G., and Dean, C. (1999) The DIF1 gene of Arabidopsis is required for meiotic chromosome segregation and belongs to the REC8/RAD21 cohesin gene family. Plant J 19, 463–472.

Birkenbihl, R.P., Jach, G., Saedler, H., and Huijser, P. (2005) Functional dissection of the plant-specific SBP-domain: overlap of the DNA-binding and nuclear localization domains. J Mol Biol 352, 585-596.

Bowman, J.L., Smyth, D.R., Meyerowitz, E.M. (1989) Genes directing flower development in Arabidopsis. Plant Cell 1, 37–52.

Canales, C., Bhatt, A.M., Scott, R., and Dickinson, H. (2002) EXS, a putative LRR receptor kinase, regulates male germline cell number and tapetal identity and promotes seed development in *Arabidopsis*. Curr Biol 12, 1718–1727.

Cardon, G.H., Höhmann, S., Nettesheim, K., Saedler, H., and Huijser, P. (1997) Functional analysis of the *Arabidopsis thaliana* SBP-box gene *SPL3*: a novel gene involved in the floral transition. Plant J 12, 367–377.

Cardon, G.H., Höhmann, S., Klein, J., Nettesheim, K., Saedler, H., and Huijser, P. (1999) Molecular characterisation of the Arabidopsis SBP-box genes. Gene 237, 91–104.

Cheng, H., Qin, L., Lee, S., Fu, X., Richards, D.E., Cao, D., Luo, D., Harberd, N.P. and Peng, J. (2004) Gibberellin regulates Arabidopsis floral development via suppression of DELLA protein function. Development 131, 1055-1064.

Chiang, H.H., Hwang, I., and Goodman, H.M. (1995) Isolation of the *Arabidopsis GA4* locus. Plant Cell 7, 195-201.

Choe, S., Dilkes, B.P., Gregory, B.D., Ross, A.S., Yuan, H., Noguchi, T., Fujioka, S., Takatsuto, S., Tanaka, A., Yoshida, S., Tax, F.E., and Feldmann, K.A. (1999) The Arabidopsis *dwarf1* mutant is defective in the conversion of 24-methylenecholesterol to campesterol in brassinosteroid biosynthesis. Plant Physiol 119, 897-907.

Chory, J., Nagpal, P., and Peto, C.A. (1991) Phenotypic and genetic analysis of *det2,* a new mutant that affects light-regulated seedling development in *Arabidopsis*. Plant Cell 3, 445-459.

Clontech (1999) MATCHMAKER GAL4 two-hybrid system 3 and libraries user manual.

Clough, S.J., and Bent, A.F. (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. Plant J 16, 735-743.

Clouse, S.D., Langford, M., and McMorris, T.C. (1996) A brassinosteroid-insensitive mutant in *Arabidopsis thaliana* exhibits multiple defects in growth and development. Plant Physiol 11, 671-678.

Colombo, N., and Favret, E.A. (1996) The effect of gibberellic acid on male fertility in bread wheat. Euphytica 91, 297-303.

Cowling, R.J., Kamiya, Y., Seto, H. and Harberd, N.P. (1998) Gibberellin dose-response regulation of *GA4* gene transcript levels in Arabidopsis. Plant Physiol 117, 1195-1203.

Davies, B., Egea-Cortines, M., de Andrade Silva, E., Saedler, H., and Sommer, H. (1996) Multiple interactions amongst floral homeotic MADS box proteins. EMBO J 15, 4330-4343.

Dawson, J., Sözen, E., Vizir, I., Van Waeyenberge, S., Wilson, Z.A., and Mulligan, B.J. (1999). Characterization and genetic mapping of a

mutation (*ms35*) which prevents anther dehiscence in *Arabidopsis thaliana* by affecting secondary wall thickening in the endothecium. New Phytol 144, 213–222.

Dill, A., Thomas, S.G., Hu, J., Steber, C.M. and Sun, T-P. (2004) The Arabidopsis F-box protein SLEEPY1 targets gibberellin signaling repressors for gibberellin-induced degradation. Plant Cell 16, 1392-1405.

Dugas, D.V., Bartel, B. (2004) MicroRNA regulation of gene expression in plants. Curr Opin Plant Biol 7, 512–520.

Edwards, K., Johnstone, C., and Thompson, C. (1991) A simple and rapid method for the preparation of plant genomic DNA for PCR analysis. Nucleic Acids Res 19, 1349-1352.

Feys, B.J., Benedetti, C.E., Penfold, C.N., and Turner, J.G. (1994) Arabidopsis mutants selected for resistance to the phytotoxin coronatine are male sterile, insensitive to methyl jasmonate, and resistant to a bacterial pathogen. Plant Cell 6, 751-759.

Filardo, F.F., and Swain, S.M. (2003) SPYing on GA signaling and plant development. J Plant Growth Regul 22, 163-175.

Finley, Jr.R.L., and Brent, R. (1994) Interaction mating reveals binary and ternary connections between *Drosophila* cell cycle regulators. Proc Natl Acad Sci USA 91, 12980-12984.

Fleet, C.M., and Sun, T.P. (2005) A DELLAcate balance: the role of gibberellin in plant organogenesis. Curr Opin Plant Biol 8, 77-85.

Fridborg, I., Kuusk, S., Moritz, T. and Sundberg, E. (1999) The Arabidopsis dwarf mutant *shi* exhibits reduced gibberellin responses conferred by overexpression of a new putative zinc finger protein. Plant Cell 11, 1019-1032.

Fu, X., Richards, D.E., Ait-ali, T., Hynes, L.W., Ougham, H., Peng, J., and Harberd, N.P. (2002) Gibberellin-mediated proteasome-dependent degradation of the Barley DELLA protein SLN1 repressor. Plant Cell 14, 3191-3200.

Fu, X., and Harberd, N.P. (2003) Auxin promotes *Arabidopsis* root growth by modulating gibberellin response. Nature 421, 740–743.

Fu, X., Richards, D.E., Fleck, B., Xie, D., Burton, N. and Harberd, N.P. (2004) The Arabidopsis mutant sleepy1^{gar2-1} protein promotes plant growth by increasing the affinity of the SCF^{SLY1} E3 ubiquitin ligase for DELLA protein substrates. Plant Cell 16, 1406-1418.

Fukazawa, J., Sakai, T., Ishida, S., Yamaguchi, I., Kamiya, Y. and Takahashi, Y. (2000) REPRESSION OF SHOOT GROWTH, a bZIP transcriptional activator, regulates cell elongation by controlling the level of gibberellins. Plant Cell 12, 901-915.

Gaillard, C., Moffatt, B.A., Blacker, M., and Laloue, M. (1998) Male sterility associated with APRT deficiency in *Arabidopsis thaliana* results from a mutation in the gene *APT1*. Mol Gen Genet 257, 348-353.

Ge, X., Dietrich, C., Matsuno, M., Li, G., Berg, H., and Xia, Y. (2005) An arabidopsis aspartic protease functions as an anti-cell-death component in reproduction and embryogenesis. EMBO Rep 6, 282-288.

Goldberg, R.B., Beals, T.P., and Sanders, P.M. (1993) Anther development: basic principles and practical applications. Plant Cell 5, 1217–1229.

Gubler, F., Raventós, D., Keys, M., Watts, R., Mundy, J., and Jacobsen, J.V. (1999) Target genes and regulatory domains of the GAMYB transcriptional activator in cereal aleurone. Plant J 17, 1-9.

Hedden, P., and Phillips, A.L. (2000) Gibberellin metabolism: new insights revealed by the genes. Trends Plant Sci 5, 523-530.

Higginson, T., Li, S.F., and Parish, R.W. (2003) *AtMYB103* regulates tapetum and trichome development in *Arabidopsis thaliana*. Plant J 35, 177–192.

Huang, S., Cerny, R.E., Qi, Y., Bhat, D., Aydt, C.M., Hanson, D.D., Malloy, K.P. and Ness, L.A. (2003) Transgenic studies on the involvement of cytokinin and gibberellin in male development. Plant Physiol 131, 1270-1282.

Huijser, P., Klein, J., Lönnig, W.E., Meijer, H., Saedler, H., and Sommer, H. (1992). Bracteomania, an inflorescence anomaly, is caused by the loss of function of the MADS-box gene SQUAMOSA in Antirrhinum majus. EMBO J. 11, 1239–1249.

Ikeda, A., Ueguchi-Tanaka, M., Sonoda, Y., Kitano, H., Koshioka, M., Futsuhara, Y., Matsuoka, M., and Yamaguchi, J. (2001) *slender* rice, a constitutive gibberellin response mutant, is caused by a null mutation of the *SLR1* gene, an ortholog of the height-regulating gene *GAI*/*RGA*/*RHT*/*D8*. Plant Cell 13, 999-1010.

Immink, R.G.H., Gadella Jr., T.W.J., Ferrario, S., Busscher, M., and Angenent, G.C. (2002) Analysis of MADS box protein-protein interactions in living plant cells. Proc Natl Acad Sci USA 99, 2416-2421.

Ishida, S., Fukazawa, J., Yuasa, T., and Takahashi, Y. (2004) Involvement of 14-3-3 signaling protein binding in the functional regulation of the transcriptional activator REPREESION OF SHOOT GROWTH by gibberellins. Plant Cell 16, 2641-2651.

Ishiguro, S., Kawai-Oda, A., Ueda, J., Nishida, I. and Okada, K. (2001) The *DEFECTIVE IN ANTHER DEHISCENCE1* gene encodes a novel phospholipase A1 catalyzing the initial step of jasmonic acid biosynthesis, which synchronizes pollen maturation, anther

dehiscence, and flower opening in Arabidopsis. Plant Cell 13, 2191-2209.

Ito, T., Wellmer, F., Yu, H., Das, P., Ito, N., Alves-Ferreira, M., Riechmann, J.L., and Meyerowitz, E.M. (2004) The homeotic protein AGAMOUS controls microsporogenesis by regulation of *SPOROCYTELESS*. Nature 430, 356–360.

Itoh, H., Ueguchi-Tanaka, M., Sato, Y., Ashikari, M., and Matsuoka, M. (2002) The gibberellin signaling pathway is regulated by the appearance and disappearance of SLENDER RICE1 in nuclei. Plant Cell 14, 57-70.

Izhaki, A., Borochov, A., Zamski, E., and Weiss, D. (2002) Gibberellin regulates post-microsporogenesis processes in petunia anthers. Physiol Plant 115, 442-447.

Jach, G., Binot, E., Frings, S., Luxa, K., and Schell, J. (2001) Use of red fluorescent protein from *Discosoma* sp. (dsRED) as a reporter for plant gene expression. Plant J 28, 483-491.

Jacobsen, S.E., and Olszewski, N.E. (1991) Characterization of the arrest in anther development associated with gibberellin deficiency of the *gib-1* mutant of tomato. Plant Physiol 97, 409-414.

Jacobsen, S.E., and Olszewski, N.E. (1993) Mutations at the SPINDLY locus of Arabidopsis alter gibberellin signal transduction. Plant Cell 5, 887-896.

Kidner, C.A., and Martienssen, R.A. (2005) The developmental role of microRNA in plants. Curr Opin Plant Biol 8, 38-44.

Klein, J., Saedler, H., and Huijser, P. (1996). A new family of DNA binding proteins includes putative transcriptional regulators of the *Antirrhinum majus* floral meristem identity gene *SQUAMOSA*. Mol Gen Genet 250, 7–16.

Koornneef, M., and Van der Veen, J.H. (1980) Induction and analysis of gibberellin-sensitive mutants in *Arabidopsis thaliana* (L.) Heynh. Theor Appl Genet 58, 257-263.

Krizek, B.A., and Meyerowitz, E,M. (1996) The *Arabidopsis* homeotic genes *APETALA3* and *PISTILLATA* are sufficient to provide the B class organ identity function. Development 122, 11–22.

Lännenpää, M., Jänönen, I., Hölttä-Vuori, M., Gardemeister, M., Porali, I., and Sopanen, T. (2004) A new SBP-box gene *BpSPL1* in silver birch (*Betula pendula*). Physiol Plant 120, 491-500.

Lee, S., Cheng, H., King, K.E., Wang, W., He, Y., Hussain, A., Lo, J., Harberd, N.P., and Peng, J. (2002) Gibberellin regulates *Arabidopsis* seed germination via *RGL2*, a *GAI/RGA*-like gene whose expression is up-regulated following imbibition. Genes Dev 16, 646-658.

Lincoln, C., Britton, J.H., and Estelle, M. (1990) Growth and development of the *axr1* mutants of *Arabidopsis*. Plant Cell 2, 1071-1080.

Ma, H. (2005) Molecular genetic analyses of microsporogenesis and microgametogenesis in flowering plants. Annu Rev Plant Biol 56, 393-434.

Magome, H., Yamaguchi, S., Hanada, A., Kamiya, Y. and Oda, K. (2004) *dwarf and delayed-flowering 1*, a novel *Arabidopsis* mutant deficient in gibberellin biosynthesis because of overexpression of a putative AP2 transcription factor. Plant J 37, 720-729.

McGinnis, K.M., Thomas, S.G., Soule, J.D., Strader, L.C., Zale, J.M., Sun, T.P., and Steber, C.M. (2003) The Arabidopsis *SLEEPY1* gene encodes a putative F-box subunit of an SCF E3 ubiquitin ligase. Plant Cell 15, 1120-1130.

Meier, C., Bouquin, T., Nielsen, M.E., Raventos, D., Mattsson, O., Rocher, A., Schomburg, F., Amasino, R.M., and Mundy, J. (2001) Gibberellin

response mutants identified by luciferase imaging. Plant J 25, 509-519.

Mercier, R., Vezon, D., Bullier, E., Motamayor, J.C., Sellier, A., Lefevre, F., Pelletier, G., and Horlow, C. (2001) SWITCH1 (SWI1): a novel protein required for the establishment of sister chromatid cohesion and for bivalent formation at meiosis. Genes Dev 15, 1859–1871.

Millar, A.A., and Gubler, F., (2005) The Arabidopsis *GAMYB-like* genes, *MYB33* and *MYB65*, are microRNA-regulated genes that redundantly facilitate anther development. Plant Cell 17, 705-721.

Mizukami, Y., and Ma, H. (1992) Ectopic expression of the floral homeotic gene *AGAMOUS* in transgenic *Arabidopsis* plants alters floral organ identity. Cell 71, 119–131.

Moreno, M.A., Harper, L.C., Krueger, R.W., Dellaporta, S.L., and Freeling, M. (1997) Liguleless1 encodes a nuclear-localized protein required for induction of ligules and auricles during maize organogenesis. Genes Dev 11, 616–628.

Murray, F., Kalla, R., Jacobsen, J.V., and Gubler, F. (2003) A role for HvGAMYB in anther development. Plant J 33, 481-491.

Nakazawa, M., Yabe, N., Ichikawa, T., Yamamoto, Y.Y., Yoshizumi, T., Hasunuma, K., and Matsui, M. (2001) DFL1, an auxin-responsive GH3 gene homologue, negatively regulates shoot cell elongation and lateral root formation, and positively regulates the light response of hypocotyle length. Plant J 25, 213-221.

Okushima, Y., Mitina, I., Quach, H.L., and Theologis, A. (2005) AUXIN REPOSNE FACTOR2 (ARF2): a pleiotropic developmental regulator. Plant J 43, 29-46.

Olszewski, N., Sun, T.P., and Gubler, F. (2002) Gibberellin Signaling: biosynthesis, catabolism, and response pathways. Plant Cell 14, S61-S80.

Park, M.Y., Wu, G., Gonzalez-Sulser, A., Vaucheret, H., and Poethig, R.S. (2005) Nuclear processing and export of microRNAs in *Arabidopsis*. Proc Natl Acad Sci USA 102, 3691-3696.

Pelaz, S., Gustafson-Brown, C., Kohalmi, S.E., Crosby, W.L., and Yanofsky, M.F. (2001) APETALA1 and SEPALLATA3 interact to promote flower development. Plant J 26, 385-394.

Peng, J., Carol, P., Richards, D.E., King, K.E., Cowling, R.J., Murphy, G.P., Harberd, N.P. (1997) The *Arabidopsis GAI* gene defines a signaling pathway that negatively regulates gibberellin responses. Genes Dev 11, 3194-3205.

Peng, J. Richards, D.E., Moritz, T., Caño-Delgado, A. and Harberd, N.P. (1999) Extragenic suppressors of the Arabidopsis *gai* mutation alter the dose-response relationship of diverse gibberellin responses. Plant Physiol 119, 1199-1208.

Perazza, D., Vachon, G., and Herzog, M. (1998) Gibberellins promote trichome formation by up-regulating *GLABROUS1* in Arabidopsis. Plant Physiol 117, 375–383.

Richards, D.E., King, K.E., Ait-ali,T., and Harberd, N.P. (2001) How gibberellin regulates plant growth and development: A molecular genetic analysis of gibberellin signaling. Annu Rev Plant Biol 52, 67-88.

Riechmann, J.L., Heard, J., Martin, G., Reuber, L., Jiang, Z.C., Keddie, J., Adam, L., Pineda, O., Ratcliffe, O.J., Samaha, R.R., Creelman, R., Pilgrim, M., Broun, P., Zhang, J.Z., Ghandehari, D., Sherman, B.K., Yu, G.L. (2000) Arabidopsis transcription factors: genome-wide comparative analysis among eukaryotes. Science 290, 2105-2110.

Rieu, I., Wolters-Arts, M., Derksen, J., Mariani, C., and Weterings, K. (2003) Ethylene regulates the timing of anther dehiscence in tobacco. Planta 217, 131–137.

Rhoades, M.W., Reinhart, B.J., Lim, L.P., Burge, C.B., Bartel, B., and Bartel, D.P. (2002) Prediction of plant microRNA targets. Cell 110, 513–520.

Ross, J.J. (1994) Recent advances in the study of gibberellin mutants. Plant Growth Regul 15, 193-206.

Ross, J.J., O'neill, D.P., Smith, J.J., Kerckhoffs, L., Huub J., and Elliott, R.C. (2000) Evidence that auxin promotes gibberellin A1 biosynthesis in pea. Plant J 21, 547-552.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press).

Sanders, P.M., Bui, A.Q., Weterings, K., McIntire, K.N., Hsu, Y.C., Lee, P.Y., Truong, M.T., Beals, T.P., and Goldberg, R.B. (1999) Anther developmental defects in *Arabidopsis thaliana* male-sterile mutants. Sex Plant Reprod 11, 297–322.

Sanders, P.M., Lee, P.Y., Biesgen, C., Boone, J.D., Beals, T.P., Weiler, E.W., and Goldberg, R.B. (2000) The Arabidopsis *DELAYED DEHISCENCE1* gene encodes an enzyme in the jasmonic acid synthesis pathway. Plant Cell 12, 1041-1062.

Sasaki, A., Itoh, H., Gomi, K., Ueguchi-Tanaka, M., Ishiyama, K., Kobayashi, M., Jeong, D.H., An, G., Kitano, H., Ashikari, M., and Matsuoka, M. (2003) Accumulation of phosphorylated repressor for gibberellin signaling in an F-box mutant. Science 299, 1896-1898.

Sawhney, V.K., and Shukla, A. (1994) Male sterility in flowering plants: are plant growth substances involved? Amer J Bot 81, 1640-1647.

Schiefthaler, U., Balasubramanian, S., Sieber, P., Chevalier, D., Wisman, E.,

and Schneitz, K. (1999) Molecular analysis of *NOZZLE*, a gene involved in pattern formation and early sporogenesis during sex organ development in *Arabidopsis thaliana*. Proc Natl Acad Sci USA 96, 11664–11669.

Schmid, M., Uhlenhaut, N.H., Godard, F., Demar, M., Bressan, R., Weigel, D., and Lohmann, J.U. (2003) Dissection of floral induction pathways using global expression analysis. Development 130, 6001-6012.

Schwab, R., Palatnik, J. F., Riester, M., Schommer, C., Schmid, M., and Weigel, D. (2005) Specific effects of microRNAs on the plant transcriptome. Dev Cell 8, 517–527.

Scott, R.J., Spielman, M., and Dickinson, H.G. (2004) Stamen structure and function. Plant Cell 16, S46-S60.

Silverstone, A.L., Ciampaglio, C.N., and Sun, T.P. (1998) The Arabidopsis *RGA* gene encodes a transcriptional regulator repressing the gibberellin signal transduction pathway. Plant Cell 10, 155-170.

Smyth, D.R., Bowman, J.L., and Meyerowitz, E.M. (1990) Early flower development in *Arabidopsis*. Plant Cell 2, 755-767.

Sorensen, A., Guerineau, F., Canales-Holzeis, C., Dickinson, H.G. and Scott, R.J. (2002) A novel extinction screen in *Arabidopsis thaliana* identifies mutant plants defective in early microsporangial development. Plant J 29, 581-594.

Sorensen, A.M., Kröber, S., Unte, U.S., Huijser, P., Dekker, K., and Saedler, H. (2003) The *Arabidopsis ABORTED MICROSPORES* (*AMS*) gene encodes a MYC class transcription factor. Plant J 33, 413–423.

Staswick, P.E., Serban, B., Rowe, M., Tiryaki, I., Maldonado, M.T., Maldonado, M.C., and Suza, W. (2005) Characterization of an Arabidopsis enzyme family that conjugates amino acids to indole-3-acetic acid. Plant Cell 17, 616-627.

Steber, C.M., Cooney, S.E., and McCourt, P. (1998) Isolation of the GA-response mutant *sly1* as a suppressor of *ABI1-1* in *Arabidopsis thaliana*. Genetics 149, 509-521.

Steiner-Lange, S., Unte, U.S., Eckstein, L., Yang, C., Wilson, Z.A., Schmelzer, E., Dekker, K., and Saedler, H. (2003) Disruption of *Arabidopsis thaliana MYB26* results in male sterility due to non-dehiscent anthers. Plant J 34, 519-528.

Stintzi, A., and Browse, J. (2000) The *Arabidopsis* male-sterile mutant, *opr3*, lacks the 12-oxophytodienoic acid reductase required for jasmonate synthesis. Proc Natl Acad Sci USA 97, 10625-10630.

Stone, J.M., Liang, X., Nekl, E.R., and Stiers, J.J. (2005) Arabidopsis *AtSPL14*, a plant-specific SBP-domain transcription factor, participates in plant development and sensitivity to fumonisin B1. Plant J 41, 744-754.

Swain, S.M., Tseng, T.S., and Olszewski, N.E. (2001) Altered expression of *SPINDLY* affects gibberellin response and plant development. Plant Physiol 126, 1174-1185.

Szekeres, M., Németh, K., Koncz-Kálmán, Z., Mathur, J., Kauschmann, A., Altmann, T., Rédei, G.P., Nagy, F., Schell, J., and Koncz, C. (1996) Brassinosteroids rescue the deficiency of CYP90, a cytochrome P450, controlling cell elongation and de-etiolation in Arabidopsis. Cell 85, 171-182.

The Arabidopsis Genome Initiative (2001) Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. Nature 408, 796-814.

Theissen, G., and Saedler, H. (2001) Plant biology: floral quartets. Nature 409, 469-471.

Tiryaki, I., and Staswick, P.E. (2002) An Arabidopsis mutant defective in jasmonate response is allelic to the auxin-signaling mutant *axr1*.

Plant Physiol 130, 887–894.

Tyler, L., Thomas, S.G., Hu, J., Dill, A., Alonso, J.M., Ecker, J.R., and Sun, T.P. (2004) DELLA proteins and gibberellin-regulated seed germination and floral development in Arabidopsis. Plant Physiol. 135, 1008-1019.

Ueguchi-Tanaka, M., Ashikari, M., Nakajima, M., Itoh, H., Katoh, E., Kobayashi, M., Chow, T., Hsing, Y.C., Kitano, H., Yamaguchi, I., and Matsuoka, M. (2005) *GIBBERELLIN INSENSITIVE DWARF1* encodes a soluble receptor for gibberellin. Nature 437: 693-698.

Unte, U.S., Sorensen, A.M., Pesaresi, P., Gandikota, M., Leister, D., Saedler, H., and Huijser, P. (2003) *SPL8*, an SBP-box gene that affects pollen sac development in Arabidopsis. Plant Cell 15, 1009-1019.

Wang, H., Caruso, L.V., Downie, A.B., and Perry, S.E. (2004) The embryo MADS domain protein AGAMOUS-like 15 directly regulates expression of a gene encoding an enzyme involved in gibberellin metabolism. Plant Cell 16, 1206-1219.

Wang, H., Nussbaum-Wagler, T., Li, B., Zhao, Q., Vigouroux, Y., Faller, M., Bomblies, K., Lukens, L., and Doebley, J.F. (2005) The origin of the naked grains of maize. Nature 436, 714-719.

Werner, T., Motyka, V., Laucou, V., Smets, R., Van Onckelen, H., and Schmülling, T. (2003) Cytokinin-deficient transgenic Arabidopsis plants show multiple developmental alterations indicating opposite functions of cytokinins in the regulation of shoot and root meristem activity. Plant Cell 15, 2532-2550.

Wilson, Z.A., Morroll, S.M., Dawson, J., Swarup, R., and Tighe, P.J. (2001) The *Arabidopsis MALE STERILITY1* (*MS1*) gene is a transcriptional regulator of male gametogenesis, with homology to the PHD-finger family of transcription factors. Plant J 28, 27–39.

Xie, D., Feys, B.F., James, S., Nieto-Rostro, M., and Turner, J.G., (1998) *COI1*: an *Arabidopsis* gene required for jasmonate-regulated defense and fertility. Science 280, 1091-1094.

Xu, Y.L., Li, L., Wu, K., Peeters, A.J.M., Gage, D.A., and Zeevaart, J.A.D. (1995) The *GA5* locus of *Arabidopsis thaliana* encodes a multifunctional gibberellin 20-oxidase: Molecular cloning and funcitonal expression. Proc Natl Acad Sci USA 92, 6640-6644.

Xu, Y.L., Li, L., Gage, D.A., and Zeevaart, J.A.D. (1999) Feedback regulation of *GA5* expression and metabolic engineering of gibberellin levels in Arabidopsis. Plant Cell 11, 927-936.

Yamasaki, K., Kigawa, T., Inoue, M., Tateno, M., Yamasaki, T., Yabuki, T., Aoki, M., Seki, E., Matsuda, T., Nunokawa, E., Ishizuka, Y., Terada, T., Shirouzu, M., Osanai, T., Tanaka, A., Seki, M., Shinozaki, K., and Yokoyama, S. (2004) A novel zinc-binding motif revealed by solution structures of DNA-binding domains of *Arabidopsis* SBP-family transcription factors. J Mol Biol 337, 49-63.

Yang, H.Q., Tang, R.H., and Cashmore, A.R. (2001) The signaling mechanism of Arabidopsis CRY1 involved direct interaction with COP1. Plant Cell 13, 2573-2587.

Yang, S.L., Xie, L.F., Mao, H.Z., Puah, C.S, Yang, W.C., Jiang, L., Sundaresan, V., and Ye, D. (2003) *TAPETUM DETERMINANT1* is required for cell specialization in the Arabidopsis anther. Plant Cell 15, 2792–2804.

Yang, S.L., Jiang, L., Puah, C.S., Xie, L.F., Zhang, X.Q., Chen, L.Q., Yang, W.C., and Ye, D. (2005) Overexpression of *TAPETUM DETERMINANT1* alters the cell fates in the Arabidopsis carpel and tapetum via genetic interaction with *EXCESS MICROSPOROCYTES1*/*EXTRA SPOROGENOUS CELLS*. Plant Physiol 139, 186–191.

Yang, W.C., Ye, D., Xu, J., and Sundaresan, V. (1999) The *SPOROCYTELESS* gene of *Arabidopsis* is required for initiation of sporogenesis and encodes a novel nuclear protein. Genes Dev 13, 2108–2117.

Zhao, D.Z., Wang, G.F., Speal, B., and Ma, H. (2002) The *EXCESS MICROSPOROCYTES1* gene encodes a putative leucine-rich repeat receptor protein kinase that controls somatic and reproductive cell fates in the *Arabidopsis* anther. Genes Dev 16, 2021–2031.

Zhao, Y., Dai, X., Blackwell, H.E., Schreiber, S.L., and Chory, J. (2003) SIR1, an upstream component in auxin signaling identified by chemical genetics. Science 301, 1107-1110.

Zhu, Q.H., Ramm, K., Shivakkumar, R., Dennis, E.S., and Upadhyaya, N.M., (2004) The *ANTHER INDEHISCENCE1* gene encoding a single MYB domain protein is involved in anther development in rice. Plant Physiol 135, 1514-1525.

Zik, M., and Irish, V.F. (2003) Global identification of target genes regulated by *APETALA3* and *PISTILLATA* floral homeotic gene action. Plant Cell 15, 207-222.

Zimmermann, P., Hirsch-Hoffmann, M., Hennig, L., and Gruissem, W. (2004) GENEVESTIGATOR, Arabidopsis microarray database and analysis toolbox. Plant Physiol 136, 2621-2632.

8. Appendix

Appendix A. Transgenic plants generated this study.

Stock	Construct	Genetic Background	Note
70030	pZY102	*spl8-1*	T_2 seeds, *35S::SPL8*#2
70031	pZY102	*spl8-1*	T_2 seeds, *35S::SPL8*#3
70032	pZY102	*spl8-1*	T_2 seeds, *35S::SPL8*#4
70033	pZY102	*spl8-1*	T_2 seeds, *35S::SPL8*#5
70034	pZY102	*spl8-1*	T_2 seeds, *35S::SPL8*#6
70028	pZY102	*spl8-1*	T_3 seeds, *35S::SPL8*#1, homozygous
70029	pZY102	*spl8-1*	T_4 seeds, *35S::SPL8*#1, homozygous
70036	pZY102	*spl8-1*	T_5 seeds, *35S::SPL8*#1, homozygous
70099	pZY102	wild-type	F_2, homozygous from cross wild-type + 70028
70100	pZY102	wild-type	F_2, homozygous from cross wild-type + 70028
70101	pZY102	wild-type	F_2, homozygous from cross wild-type + 70028
70021	pZY112	wild-type	T_4 seeds, *35S::SBP-box*, homozygous
70022	pZY104	wild-type	T_4 seeds, *35S::SPL8*(S63D), homozygous
70115	pZY108	wild-type	T_2 seeds, *35S::SPL8*(S63A)-dGFP
70116	pZY109	wild-type	T_2 seeds, 35S::SPL8(S63D)-dGFP
70117	pZY109	wild-type	T_2 seeds, *35S::SPL8*(S63D)-dGFP
70118	pZY109	wild-type	T_2 seeds, *35S::SPL8*(S63D)-dGFP
70119	pZY101	*spl8-1*	T_2 from T_1#1, *pSPL8::SPL8*, homozygous
70126	pZY101	*spl8-1*	T_2 from T_1#2, *pSPL8::SPL8*, homozygous
70134	pZY101	*spl8-2*	T_2 from T_1#16, *pSPL8::SPL8*, homozygous
70137	pZY101	*spl8-2*	T_2 from T_1#6, *pSPL8::SPL8*, homozygous
70141	pZY101	*spl8-2*	T_2 from T_1#5, *pSPL8::SPL8*, homozygous
70147	pZY101	*spl8-3*	T_2 from T_1#4, *pSPL8::SPL8*, homozygous
70152	pZY101	*spl8-3*	T_2 from T_1#9, *pSPL8::SPL8*, homozygous

Appendix B. Constructs generated in this study

Construct No.	Backbone-Insertion	sequencing primer pair	DNA template	PCR primers
pZY101	pGJ2148-SPL8 genomic fragment	ZY01-ZY05 SH134, SH181	from BAC	NA
pZY102	pGJ2171-*SPL8*	ZY10/ZY09/GC354	pRB504	ZY06/ZY08
pZY103	pGJ2171-*SPL8* S63A	ZY10/ZY09	pRB521	
pZY104	pGJ2171-*SPL8* S63D	ZY10/ZY09	pRB522	
pZY105	pGJ2171-*SPL8* S49A	ZY10/ZY09/GC354	pRB536	
pZY107	pGJ2171-*SPL8*:dGFP	ZY09/GC326	two steps cloning: ZY06+ZY07 (template pRB504/521/522), insert into pGJ619/*Nco*I Digest with *Nco*I&*Xba*I Insert ino GJ2171	
pZY108	pGJ2171-*SPL8* S63A :dGFP	ZY10/GC354 /GC326		
pZY109	pGJ2171-*SPL8* S63D :dGFP)	ZY10/GC354 /GC326		
pZY110	pGJ2171-*SPL8* S49A :dGFP	ZY10/GC354 /GC326		
pZY112	pGJ2171-*SPL8* SBP-box	ZY10	pRB504	ZY11/ZY12
pZY115	*SPL8*	ZY17/ZY18	pRB504	ZY13/ZY14
pZY114	pGBKT7-*SPL8*Δ*nt643-1002*	ZY17	pRB504	ZY13/ZY21
pZY113	pGBKT7-*SPL8*Δ*nt1-535*	ZY17/ZY18	pRB504	ZY14/ZY15
pZY116	pGBKT7-*SPL8*Δ*nt1-418*	T7	pRB504	ZY23/ZY14
pZY118	pGBKT7- *SPL8 nt418-643*	T7	pRB504	ZY23/ZY21
pZY119	pGBKT7-*SPL8* S247A	T7/ZY18	pRB521	ZY13/ZY14
pZY120	pGBKT7-*SPL8* S247D	T7/ZY18	pRB522	ZY13/ZY14

Appendix C. Oligonucleotides used in this study

Primer	Sequence 5'-3'	Note
ZY01	GAAAGACAGGATGGCAAAAG	pZY101 sequencing
ZY02	CAACCTCCAACTTGCTACC	pZY101 sequencing
ZY03	CATGTTGCAATCCCGAGGAAGATCTC	pZY101 sequencing
ZY04	CATCTGAATTAGTCGGTAATGTGTAAC	pZY101 sequencing
ZY05	CGAAACACTACCACAGAAGGC	pZY101 sequencing
SH134	AATTAGGGAAGAAAGGAGGTGGGG	pZY101 sequencing
SH181	GTAGTCAGTTACAAGTTTATACGTGG	pZY101 sequencing
ZY40	CAAACGACAGCGTCTTCATCCA	pZY101 sequencing
ZY06	TGGTGGTCCATGGGCTTGGACTACGAATGGGATAATC	*SPL8*(*Nco*I)
ZY07	CTCCTCCACATGTATCCGCTGGAGAAAAACATTGAA	*SPL8*(*Afl*III)
ZY08	TCCTTTACCTCTAGATCAGCTGGAGAAAAACAT	*SPL8*(*Xba*I)
ZY09	ATAGATTTGTAGAGAGAGACTGGTG	CaMV35S terminator
ZY10	TCATTTCATTTGGAGAGGAC	CaMV35S promoter
ZY25	GAGTCTCATATTCACTCTCAACTC	Forward for *BAR*
ZY26	CGATCTGCTTGACTCTAGGGTC	Reverse for *BAR*
ZY11	CGAACTCGCTGAGCACGCCATGGGCTGCCAAGCA	*SPL8*(*Nco*I)
ZY12	GGTGTCTTGGGTGGCGGAGGTCTAGAGGTGA	*SPL8*(*Xba*I)
ZY13	CGAACGATACATATGTTGGACTACGAATGGGATA	*SPL8*(*Nde*I)
ZY14	CTCACCCGGGCTATCCGCTGGAGAAAAACATTGAATTTGA	*SPL8*(*Xma*I)
ZY15	GTGAGTCACATATGGCGAACTCGCTGAGCACGCCGC	*SPL8*(*Nde*I)
ZY21	CCGTCCCGGGCTATGAGTGGAATTCGCACACTTTGT	*SPL8*(*Xma*I)
ZY23	ACACATATGGATTTCACGTCCAACAGGATCG	*SPL8*(*Nde*I)
ZY17	TAATACGACTCACTATAGGGC	T7 sequencing primer
ZY18	TTTTCGTTTTAAAACCTAAGAGTC	pGBKT73' sequencing primer
ZY43	CTATTCGATGATGAAGATACCCCA	pGADT73' sequencing primer

Primer	Sequence 5'-3'	Note
ZY44	TCTACGATTCATCTGCAGCTCGAG	pGADT73' sequencing primer
ZY22	AGATGGTGCACGATGCACAG	pGADT73' sequencing primer
ZY34	AACAGTTCTGGGACTTCTGGTGG	*At5g54510* wild-type genotyping
ZY35	CCGAAGTAACACTCCGAAGAAGC	*At5g54510* wild-type genotyping
ZY36	GCGTGGACCGCTTGCTGCAACT	SALK LBb1 PCR primer
ZY37	TCTTTTCTCCTACCGTGCTACCC	*At3g09970* wild-type genotyping
ZY38	TGCCCGCTAACAACAACAGTATG	*At3g09970* wild-type genotyping
ZY39	CCCATTTGGACGTGAATGTAGACAC	GABI-CAT LB PCR primer
ZY46	CTGGTGACATTCTCCATTGATTCT	GABI-CAT 621B04 genotyping
ZY47	CCTACAGAGTATGTTCCAGAGCA	GABI-CAT 621B04 genotyping
ZY48	AAGGCTACGTTCAATTCTGTGAAAGGGATG	*At3g09970* RT-PCR primer
ZY49	CGAGCTACATTACACCACTAGACCGCTT	*At3g09970* RT-PCR primer
ZY66	TTAGGTGGCGGAGGCTGATTGGTG	*SPL8* RT-PCR primer
ZY67	ATGTTGGACTACGAATGGGATAATC	*SPL8* RT-PCR primer
ZY78	CGGTTTCTTCCTCGTGGTCAATC	*GA5* RT-PCR primer
ZY79	TGATGTGATGCTGTCCAAAAGC	*GA5* RT-PCR primer
ZY89	GAGGAAGTCTTTGACGAGGAAGC	*RGL2* RT-PCR primer
ZY90	CGGTAAATACGACGAGCCAAGG	*RGL2* RT-PCR primer
GC709	ACCAGCAAACCGTGGATTACCCTAGC	*RAN3* RT-PCR primer
GC710	ATTCCACAAAGTGAAGATTAGCGTCC	*RAN3* RT-PCR primer

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10. Lebenslauf

ADDRESSE: Kolibriweg 14, Köln, D-50829
Tel.: +49(0)221 5062 170
GEBURTSTAG: 23. November 1976, weiblich
FAMILIENSTAND: Verheiratet
NATIONALITÄT: Chinesisch

AUSBILDUNG

2002 - 2005 **MAX PLANCK INSTITUT FÜR ZÜCHTUNGSFORSCHUNG (MPIZ)**
Abteilung Molekulare Pflanzengenetik, Köln, Deutschland
Projekt: Das SBP-Box Gen *SPL8* beeinflußt die reproduktive Entwiklung und ist ein Bestandteil der Gibberellinsäure-Signalkaskade in *Arabidopsis*.

1998 – 2001 **INSTITUT FÜR GENETIK, CHINESE ACADEMY OF SCIENCES, BEIJING, CHINA**
Master of Sciences: Genetik
Projekt: Funktionelle Analyse des Barley Yellow Dwarf Virus (BYDV) Transferproteins.

1994 – 1998 **XINJIANG UNIVERSITY, URUMQI, CHINA**
Abteilung für biologische Wissenschaften,
Bachelor of Sciences: Mikrobiologie
Projekt:.Entwicklung eines Expressionsvektors zur Produktion von Verhütungsmitteln.

BERUFSERFAHRUNG

2001 – 2002 **LIU-SHEN INTELLECTUAL PROPERTY AGENCY-BIOTECH BRANCH, BEIJING, CHINA.**
Uebersetzerin