

SPL8, a local regulator in a subset of gibberellin-mediated developmental processes in Arabidopsis

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Abstract Gibberellins (GAs) are important plant growth regulators, regulating many plant developmental processes, including seed germination, root and stem elongation, rosette expansion, floral induction and anther development. The diverse effects of GAs on plant development make it critical to maintain an appropriate endogenous GA level and a fine-tuned GA signalling. Some global regulators in GA signalling have been identified but little is known about genes specifically involved in local implementation of GA signalling. Here we report that the *Arabidopsis thaliana* SBP-box gene *SQUAMOSA-PROMOTER-BINDING-PROTEIN-LIKE8* (*SPL8*) acts as a local regulator in a subset of GA-dependent developmental processes. Previous knowledge holds that *SPL8* is involved in reproductive development as deduced from its loss-of-function phenotype (Unte et al. (2003) *Plant Cell* 15:1009–1019). We now determined that constitutive overexpression of *SPL8* affects fertility due to non-dehiscent anthers, likely resulting from a constitutive GA response, suggesting a positive role of *SPL8* in GA-mediated anther development. On the other hand, *SPL8* gain- and loss-of-function mutants showed opposite responses to GA and its biosynthetic inhibitor paclobutrazol (PAC) with respect to seed germination and root elongation during

the seedling stage. Genes involved in GA biosynthesis and signalling are transcriptionally affected by altered *SPL8* expression. Our study uncovered a tissue-dependent regulatory role for *SPL8* in the response to GA signalling in plant development.

Keywords Anther development · *Arabidopsis* · Fertility · Gibberellic acid signalling · SBP-box gene

Abbreviations

GA	Gibberellic acid
PAC	Paclobutrazol
JA	Jasmonate
MeJA	Methyl jasmonate
DAG	Days after germination

Introduction

The phytohormone gibberellic acid and its derivatives (GAs) are important plant growth factors regulating many developmental processes, including seed germination, rosette leaf expansion, stem and root elongation, floral induction and anther development (Fleet and Sun 2005). The diverse and vital effects of GAs on plant development require appropriately controlled endogenous GA levels and also fine-tuned GA signal responses. Accordingly, extensive studies have been conducted, mainly through genetic screens, to get a better understanding of the GA biosynthetic and signalling pathways (Olszewski et al. 2002).

In recent years, the GA biosynthetic pathway has become well understood and GA-biosynthetic genes

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have been cloned and characterized from many plant species (Hedden and Phillips 2000). Mutants defective in these genes, like *gal-3*, show germination failure, dwarfism, late flowering and male sterility. The phenotypic alterations of GA-deficient mutants can be rescued by exogenous application of GAs (Koornneef and Van der Veen 1980).

Further efforts dedicated to comprehend GA signalling gave rise to a generally accepted derepressible-repression model (Peng et al. 1997). GA-signalling mutants supporting this model can be divided into two major classes according to their characteristic phenotypes. The first class consists of GA-insensitive mutants. They phenocopy GA-deficient mutants but cannot be rescued by exogenous GAs. These mutants carry either dominant mutations in negative regulators or recessive mutations in positive regulators of the GA signalling cascade. For instance, the Arabidopsis *ga insensitive* (*gai*) mutant allele conferred 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 of the *gai* allele rendered GAI resistance to GA-dependent derepressing effects (Dill et al. 2004; Fu et al. 2004). The group of recessive GA-insensitive mutants in Arabidopsis is represented by *sleepy1* (*sly1*; Steber et al. 1998). *SLY1* encodes an F-box protein involved in GA-dependent DELLA protein degradation (McGinnis et al. 2003; Dill et al. 2004).

The second major class of GA signalling mutants represents recessive mutations in negative regulators of GA signalling, which therefore show a constitutive response to GAs. Constitutive GA response mutants show PAC-resistant seed germination, have pale-green leaves, elongated stems and are male sterile. The Arabidopsis *spindly* (*spy*) mutants belong to this group (Jacobsen and Olszewski 1993). *SPY* 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 signalling on the whole is still elusive. Most GA signalling components characterized so far simultaneously cover a broad range of GA-regulated developmental processes, thus representing global GA response regulators. However, bioactive GAs can have dramatically different effects on plant development depending on tissue type and developmental stage (Fleet and Sun 2005). It is hence reasonable to hypothesize that plants have evolved

local acting regulatory networks to maintain appropriate GA levels and signalling in specific tissues and/or developmental stages. Such local regulators have been identified in GA biosynthesis/signalling, albeit few. The Arabidopsis homeobox protein SHOOTMERISTEMLESS inhibits a GA 20-oxidase within the shoot apical meristem to ensure a low GA level favourable for maintenance of the meristem (Hay et al. 2002). An AP2-type transcription factor, DWARF AND DELAYED-FLOWERING1 (DDF1), regulates GA biosynthesis in Arabidopsis during stem elongation and floral induction as deduced from its dominant mutant phenotype and expression pattern (Magome et al. 2004). Also Arabidopsis SHORT INTERNODES (SHI) has been proposed to be a local GA-signalling regulator as overexpression of *SHI* caused mainly shortening of the inflorescence stem and a delay of floral induction (Fridborg et al. 1999).

Here we report that the Arabidopsis SBP-domain protein *SPL8* (Cardon et al. 1999) may represent such a local acting regulator in GA signalling. Most likely being a transcription factor (Birkenbihl et al. 2005), *SPL8* has previously been shown to be required for proper micro- and megasporogenesis as deduced from its semi-sterile mutant phenotype (Unte et al. 2003). In this study, we generated *SPL8* gain-of-function transgenic lines. The phenotypic comparison of *SPL8* gain- and loss-of-function suggested that *SPL8* affects GA signalling in a tissue-specific way. Our further experimental data provided evidence to support the hypothesis that *SPL8* acts locally in a subset of GA-mediated developmental processes.

Material and methods

Plant material and growth conditions

All mutants and transgenic plants described here were derived from *Arabidopsis thaliana* ecotype Columbia-0 (wild type). Seeds of the *spy-3* mutant were obtained from the Arabidopsis stock centre (NASC). Seeds were stratified for 4 days at 4°C before sowing on soil containing a mixture of substrate and vermiculite (3:1). Seeds for growing on germination media, were surface-sterilized in 98% ethanol followed by 10% sodium hypochlorite, and rinsed with sterile water. Plated seeds were kept at 4°C for 4 days, then moved to a growth chamber. Plants were grown under controlled environmental conditions as described by Unte et al. (2003).

Generation of transgenic plants

For complementation of the *spl8* mutant, a 4,588 bp fragment covering the entire *SPL8* genomic region was derived from BAC T7I23 through a partial digest with the restriction enzymes *Bst*Z171 and *Spe*I. After Klenow-filling the ends the fragment was cloned into the *Sma*I site of the binary vector pGJ2148. To generate the 35S::*SPL8* construct, the entire *SPL8* coding sequence was subcloned into binary vector pGJ2171 which carries two copies of the Cauliflower Mosaic Virus 35S promoter. Constructs were introduced into plants using *Agrobacterium tumefaciens* strain pGV3101 (pMP90) by the floral-dip method (Clough and Bent 1998). Transgenic lines were selected on soil by spraying seedlings with 0.1% (v/v) BASTA.

Semiquantitative RT-PCR

Total RNA was extracted using the RNeasyTM kit together with the On-Column DNase system (Qiagen) according to the manufacturer's instructions. Reverse transcriptase-mediated polymerase chain reactions (RT-PCR) were performed with the Superscript II kit (Invitrogen) in the presence of α -³²PdCTP. Samples were normalized based on the radioactivity incorporated as determined by liquid scintillation counting. PCRs were performed under the following three-step cycling conditions: 24–32 PCR cycles at 94°C for 40 s, 60°C for 40 s, and 72°C for 50 s. All reactions used 1 μ l cDNA as template in 25 μ l total volume. After amplification, RT-PCR products were separated from the primers by electrophoresis in 1.2% agarose gels and quantified by phosphor imaging using ImageQuant software (Molecular Dynamics). Oligonucleotides for the detection of *SPL8* (ZY67 ATGTTGGACTACGAATGGGATAATC; ZY66 TTAGGTGGCGGAGGCTGATTGGTG), *GA5* (ZY78 CGGTTTCTTCCTCGTGGTCAATC; ZY79 TGATGTGATGCTGTCCAAAAGC), were synthesized by Invitrogen. Primer sequences for *RAN3* were as described by Unte et al. (2003), those for γ -*TIP* were described by Cowling et al. (1998).

Anther histology, pollen viability and microscopy

Determination of anther histology was performed as described by Sorensen et al. (2002). All light and scanning electron microscopy were performed according to Unte et al. (2003). Pollen viability was determined by FDA-staining (Regan and Moffatt 1990)

followed by fluorescence microscopy using the filter combination BP485 (excitation), FT510 (beamsplitter) and LP520 (barrier).

Germination and root length measurement

Sterilized seeds were spread on germination medium (GM; Murashige and Skoog medium, Duchefa) supplemented (where appropriate) with GA₃ (Sigma) or PAC (Duchefa) and kept 4 days at 4°C for stratification. Because seed storage time has a great impact on germination potential, the experiments were performed using seeds with a similar storage time from all genotypes. Germination was recorded 7 days after the plates had been moved to a growth chamber. The percentage of germination and standard deviation for each genotype were determined from six trials with 60–100 seeds per trial.

To measure root length, seedlings were grown on vertically orientated GM plates supplemented with GA₃ or PAC. Seven days after germination (DAG) the seedlings were imaged on a digital flatbed scanner. Root length was determined from the digital images using the Java based image processing program ImageJ (<http://rsb.info.nih.gov/ij/>).

Hormone and inhibitor treatment

For GA treatment of soil-grown plants, 100 μ M GA₃ (Sigma) in 0.02% Tween-20 was sprayed twice per week starting with 2-week-old seedlings. For GA or PAC treatment of flowers and seedlings, 100 μ M GA₃ or 10 μ M PAC in 0.02% Tween-20 were sprayed. Spraying 0.02% Tween-20 was always used as a mock treatment. Plant materials were collected 2 h after treatment for RNA extraction. For germination assays and root elongation measurements, 10 mM GA₃ or PAC stock solutions (in 96% ethanol) were added to autoclaved GM to their required final concentrations. GM supplemented with a comparable diluted (1:200 to 1:1000) amount of ethanol was used for the non-treated controls. 100 μ M MeJA (Sigma, USA) were used to treat mature anthers of 35S::*SPL8* transgenic plants for the dehiscence assay as described by Zhu et al. (2004).

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Results

The spatiotemporal expression of *SPL8* is important for plant development

As previously reported by Unte et al. (2003), three *spl8* mutants were identified by their semi-sterile phenotype, the severity of which decreased acropetally within the inflorescence. In addition to defects in micro- and megasporogenesis, *spl8* mutant flowers were also found to display shorter stamen filaments and a lower trichome density on their sepals, all traits known to be regulated by GAs (Perazza et al. 1998; Cheng et al. 2004). A possible role of *SPL8* in GA biosynthesis/signalling has been suggested (Unte et al. 2003).

In order to investigate this possibility, bioactive GAs were exogenously applied to *spl8* mutants but the semi-sterile mutant phenotype could not be rescued. Therefore, *SPL8* is unlikely to be directly involved in GA biosynthesis. To obtain additional clues on the possible role of *SPL8* in GA-regulated development, the *SPL8* coding sequence under the control of the strong and constitutive 35S-CaMV promoter was introduced into wild type and *spl8-1* mutants. Proper transgene expression in the transgenic lines obtained was confirmed by RT-PCR (data not shown).

In the *spl8-1* mutant background, the constitutively expressed *SPL8* transgene could not restore fertility (Fig. 1A). In fact, all of the six transgenic lines displayed an unexpected enhanced sterility, as also observed upon constitutive over-expression of *SPL8* in a wild-type background. The *SPL8* gain-of-function lines bear 10–60% fertile, elongated siliques in their fully developed primary inflorescences, whereas *spl8-1* mutant primary inflorescences produce 45–80% fertile siliques. In addition to sterility, the *SPL8* transgenic lines with either a *spl8-1* mutant or a wild-type background displayed a consistent and heritable pleiotropic abnormal phenotype. In particular the transgenics showed dwarfism, small and dark-green rosette leaves and a compact inflorescence (Fig. 1A). Furthermore, seed germination rate and root elongation were found to be reduced as discussed below. In wild-type plants, *SPL8* is predominantly expressed in inflorescence and siliques, detectable in seedlings and almost undetectable in other vegetative tissues (Fig. 1B). Therefore, we consider the pleiotropic phenotype mostly a result of ectopic expression of *SPL8* in tissues where it is normally inactive.

In contrast to this dramatic gain-of-function phenotype, the sterility of *spl8* mutants was fully rescued when *spl8-1* and *spl8-3* were transformed with *SPL8* under the control of its endogenous promoter, as part

of a 4.8 kb genomic fragment spanning the *SPL8* genomic locus. These complementation transgenic lines also displayed no discernable morphological defects (data not shown). These results strongly suggest that the spatiotemporal expression of *SPL8* is important for proper plant development.

Constitutive *SPL8* expression resulted in anther non-dehiscence

It is remarkable that both *SPL8* loss- as well as gain-of-function resulted in partial sterility. Unte et al. (2003) 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 megasporocytes degenerated at an early stage (Unte et al. 2003).

To determine whether the sterility of *SPL8* gain-of-function was due to defects in anther or ovule development, 35S::*SPL8* transgenic plants and *spl8* mutants were manually pollinated with wild-type pollen. *Spl8* mutant siliques contain many aborted ovules even after pollination with fully viable wild-type pollen (Fig. 2A), confirming the previous reported abnormal megaspore development in *spl8* (Unte et al. 2003). Unlike *spl8* mutants, the constitutive *SPL8* overexpressing plants seem not to be affected in ovule development. Their siliques showed full seed set after manual pollination with wild-type pollen (Fig. 2B).

We also compared stamen structures at stage 9 of anther development (staging according to Sanders et al. 1999) since it was shown that *spl8* mutants have abnormally short filaments and small anthers (Unte et al. 2003). In sharp contrast to *spl8* (Fig. 2E), stamens of 35S::*SPL8* (Fig. 2D) did not differ notably from wild type (Fig. 2C). This observation suggested that anther defects in 35S::*SPL8* plants start at a relatively late stage of development. Interestingly, we noticed an obvious increase of trichome density and complexity on the abaxial side of the sepals in 35S::*SPL8* flowers (Fig. 2G), which is to the opposite of *spl8* mutants (Unte et al. 2003; Fig. 2H).

A histological analysis was performed to determine at which stage the anther developmental defects appeared. It turned out that an inability of the anthers to dehisce caused the observed male sterility in 35S::*SPL8* plants. At anther stage 11, wild-type tapetum starts to degenerate and endothelial cells enlarge (Fig. 3A, B). Anthers of 35S::*SPL8* flowers show comparable endothelial enlargement and tapetal degeneration (Fig. 3E, F). The only recognizable difference is that the middle layer, originally a single cell layer between endothecium

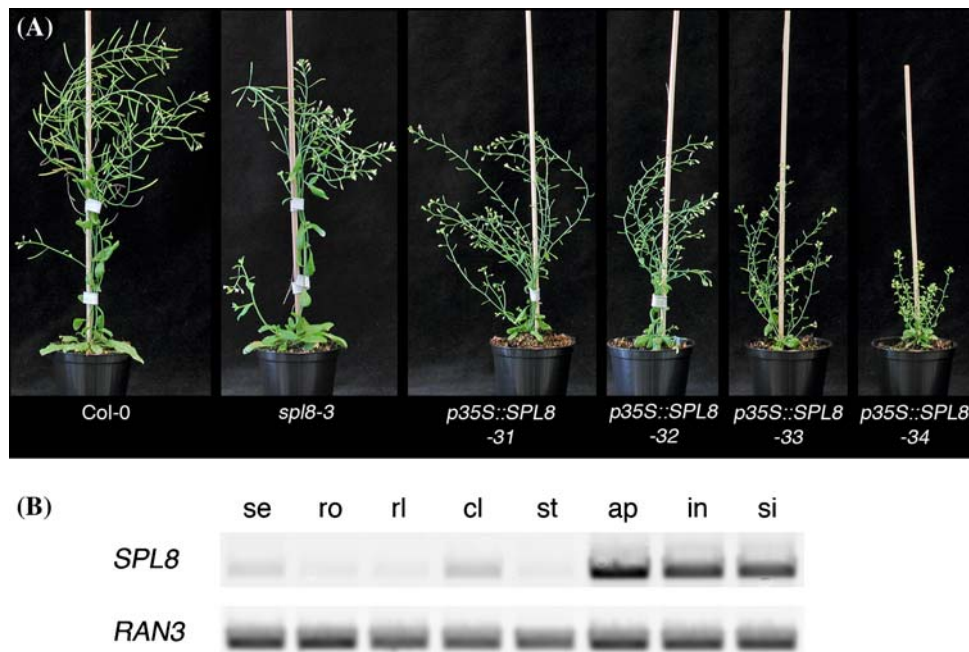


Fig. 1 The spatiotemporal expression of *SPL8* is important for proper plant development. **(A)** Independent *SPL8* overexpressing transgenic lines (*p35S::SPL8*-31 to -34) show a heritable and, although to a different degree, consistent pleiotropic phenotype, including sterility (note short and seedless siliques in comparison to Col-0 wild-type plant on the left). An *spl8* loss-of-function

mutant (*spl8-3*) is shown for comparison. **(B)** RT-PCR analysis of *SPL8* transcript levels in total RNAs isolated from different tissues and organs of wild type. *RAN3* was used as a loading control. se, seedlings; ro, roots; rl, rosette leaves; cl, cauline leaves; st, stem; ap, shoot apical region; in, inflorescence; si, siliques

and tapetum and disappearing at stage 8 in wild-type anthers, is still present in *35S::SPL8* anthers at stage 11 (Fig. 3F).

Anther stage 13 is the stage where in wild-type dehiscence happens (Sanders et al. 1999). Anther dehiscence requires secondary wall thickening through the deposition of fibrous bands (lignification) in the connective and endothelial cells (Fig. 3C, D). Subsequent enlargement of the endothelial cells together with a degenerating septum and stomium, results in rupture of the anther wall and to a release of the mature pollen grains (Fig. 3D). However, we found deposition of fibrous bands to be largely absent in connective and endothelial cells of *35S::SPL8* anthers at a comparable stage. Instead, these cells had accumulated some granular material (Fig. 3G, H). Furthermore, older *35S::SPL8* anthers showed no sign of breakage of septum and stomium. As a result, pollen grains remained trapped inside the pollen sacs. The pollen sacs of *35S::SPL8* anthers at these later stages were found to be covered with a dark-staining layer on their inside and contained, besides pollen grains, many small cellular structures of unknown origin (Fig. 3H).

Anther non-dehiscence in *35S::SPL8* is jasmonate independent

Jasmonates (JAs) are known to be a major factor affecting anther dehiscence. Mutants defective in JA biosynthesis or signalling typically show delayed anther dehiscence and pollen inviability (reviewed by Scott et al. 2004). We hence tested the effect of exogenous JA on *35S::SPL8* plants. However, application of methyl jasmonate (MeJA, converts to JA) did not counteract the failure of *35S::SPL8* anthers to dehiscence. A pollen viability assay also showed no obvious difference between wild-type (88.0%) and *35S::SPL8* plants (78.3%). Together, it is not likely that *35S::SPL8* plants are disturbed in JA biosynthesis and/or signalling.

Anther non-dehiscence in *35S::SPL8* is likely due to a constitutive GA response

GA has also been hinted as a factor affecting anther dehiscence. Studies in wheat and barley demonstrated that GA overdose and constitutive GA response cause anther non-dehiscence (Colombo and Favret 1996; Murray et al. 2003). Considering the anther defects

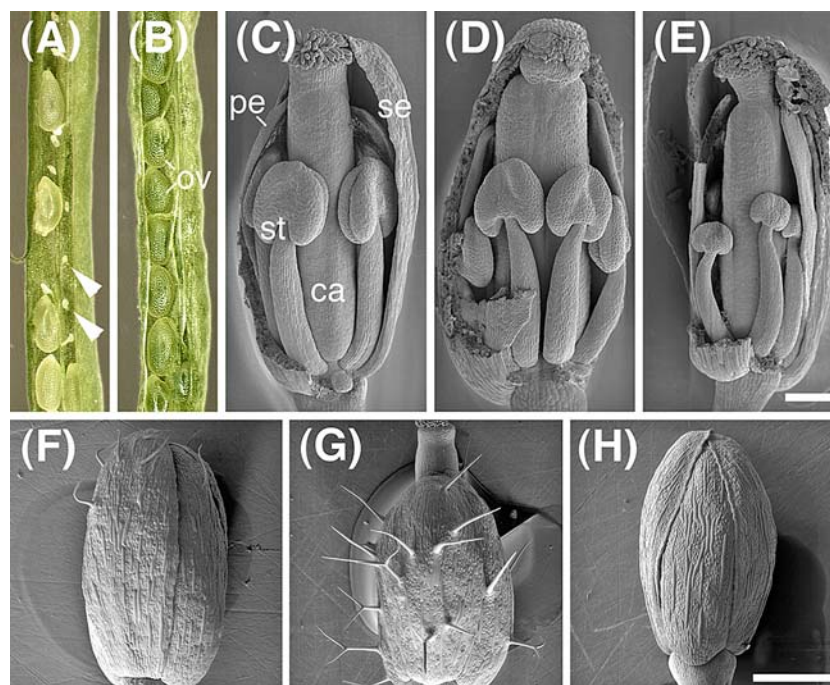


Fig. 2 Comparison of *SPL8* gain- and loss-of-function in reproductive growth. (A)–(B) Manual pollination could not prevent the abortion of many ovules (white arrowheads) in siliques of the *spl8-1* mutant (A) but resulted in full seed set of *35S::SPL8* transgenic siliques (B). (C)–(E) SEM images of floral buds at proximately anther stage 10 after the completion of meiosis in wild-type (C), and of *35S::SPL8* transgenic (D) and

spl8-1 mutant plants (E) at comparable stages. (F)–(H) Compared to wild-type (F), *35S::SPL8* transgenic sepals (G) showed an increased number of often stellate trichomes, whereas *spl8-1* mutant sepals (H) almost completely lacked trichomes. ca, carpel; ov, ovule; pe, petal; se, sepal; st, stamen. Bar = 200 μ m in C to E. Bar = 500 μ m in (F)–(H)

seen in *SPL8* gain- and loss-of-function mutants, we reasoned that anther non-dehiscence of *35S::SPL8* could be due to a constitutive GA response.

To verify that in *Arabidopsis* too, a constitutive GA response leads to non-dehiscent anthers, we examined the anther histology of GA mutant *spy-3* (Jacobsen and Olszewski 1993). Cross-sections made of *spy-3* anthers before stage 11 were indistinguishable from those of wild type (data not shown). At stage 13 *spy-3* anthers indeed showed a non-dehiscent phenotype. As mentioned before, wild-type anthers dehisce at stage 13 due to a series of events, including the enlargement of endothelial cells, deposition of fibrous bands in connective and endothelial cells, degeneration of septum and stomium (Fig. 4A, C). *Spy-3* anther at a comparable stage did show the deposition of fibrous bands but to a lesser extent (Fig. 4B, D). The degeneration of septum and stomium did not happen, which might thus be the major cause for the failure to dehisce.

Seed germination and root elongation were affected by *SPL8* gain- and loss-of-function

Except for anther development, GAs also play prominent roles in seed germination and root elongation

(Lee et al. 2002; Tyler et al. 2004; Fu and Harberd 2003).

Germination rate of *35S::SPL8* seeds was found to be 60%, a strong reduction compared to that of wild-type seeds, i.e., 98%, of same age and raised under identical conditions (Fig. 5A). A germination test was conducted to examine if the reduced germination potential of *35S::SPL8* seeds could be rescued by GAs. Concentrations of up to 50 μ M GA₃ in the medium could not, however, restore *35S::SPL8* seed germination to wild-type rates (Fig. 5A), suggesting that this phenotypic feature rather reflects GA-insensitivity than reduced endogenous GA levels.

Concerning germination potential, seeds of *spl8* mutants did not differ from wild type (Fig. 5C). Interestingly, we observed that the *spl8* allelic mutants showed an increased resistance to the GA biosynthetic inhibitor PAC during seed germination. Whereas the germination rate of wild-type seeds decreased from 93% to 20% in the presence of 10 μ M PAC, the germination rates of the *spl8* mutants remained well above 50% (Fig. 5C).

Constitutive *SPL8* overexpression also reduced root elongation significantly (Fig. 5B). At 9 DAG, roots of

Fig. 3 Male sterility of constitutive *SPL8* overexpression is due to non-dehiscent anthers. (A)–(D) Cross-sections through wild-type anthers at stage 11 when fibrous bands become visible in the endothecium (A)–(B), and stage 13 when the anther ruptures along septum and stomium due to dehiscence (C)–(D). (E)–(H) Cross-sections through *35S::SPL8* anthers at comparable stages without obvious lignification but formation of granular material in endothelial cells and still traces of middle layer visible (E)–(F). *35S::SPL8* anthers did not dehisce and pollen sacks were covered with a dark staining layer on their inside (black arrowheads) and filled with cellular structures (G)–(H). co, connective; cs, cellular structures of unknown origin; en, endothecium; ep, epidermis; fb, fibrous bands; gm, granular material; ml, middle layer; pg, pollen grain; so, stomium; sp, septum; ta, tapetum; vb, vascular bundle. Bar = 100 μ m in (A), (C), (E) and (G). Bar = 50 μ m in (B), (D), (F) and (H)

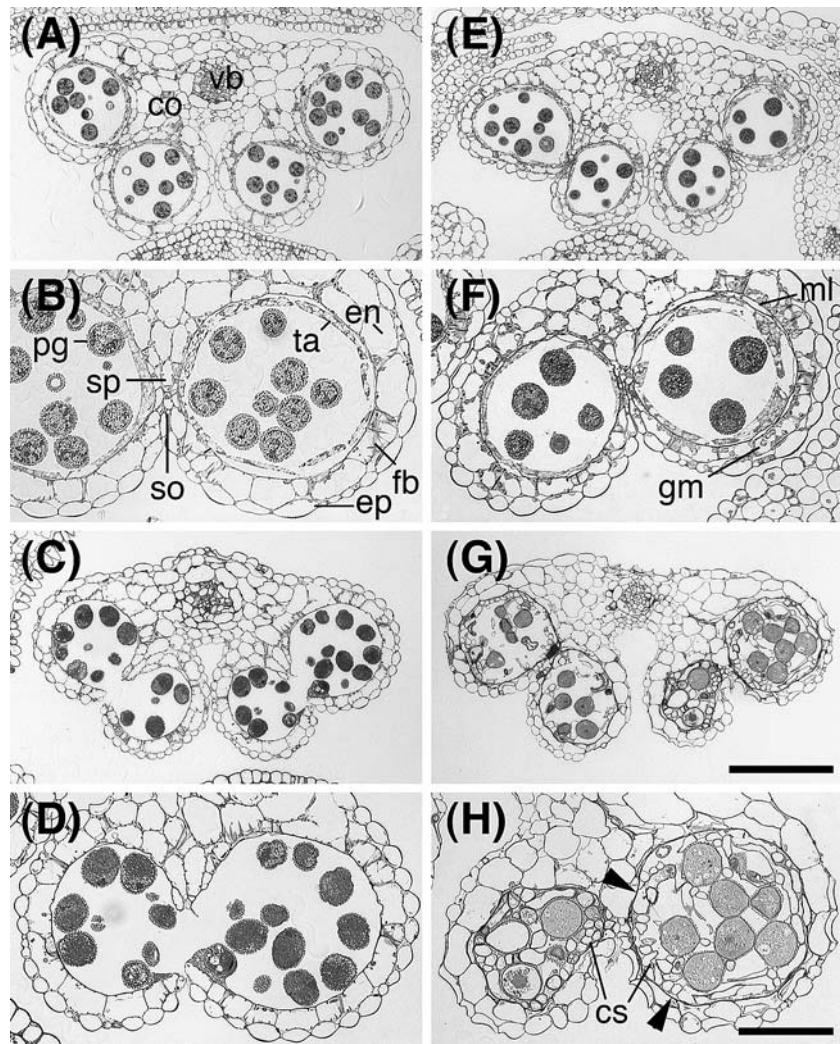
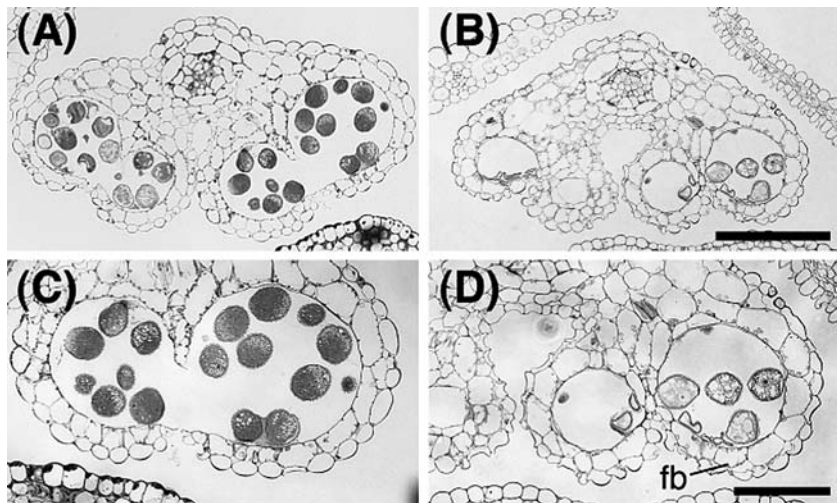


Fig. 4 Comparison of non-dehiscent *spy* mutant anther histology to wild type. Cross-sections show that the endothecium of a *spy-3* mutant anther becomes less lignified. (A) and (C), Cross-sections through a wild-type anther at stage 13 when the anther ruptures along septum and stomium. (B) and (D), Cross-sections through a *spy-3* mutant anther at a comparable stage showed less deposition of fibrous bands in the endothecium and did not rupture. fb, fibrous bands. Bar = 100 μ m in (A) and (B). Bar = 50 μ m in (C) and (D)



35S::SPL8 seedlings reached only half the length of wild type, and same as the lower germination rate, extraneously applied GA_3 did not affect root elongation

(Fig. 5B). In contrast, *spl8* mutants revealed an enhanced root growth (Fig. 5B). Constitutive *SPL8* overexpression thus conferred a certain GA -insensitivity

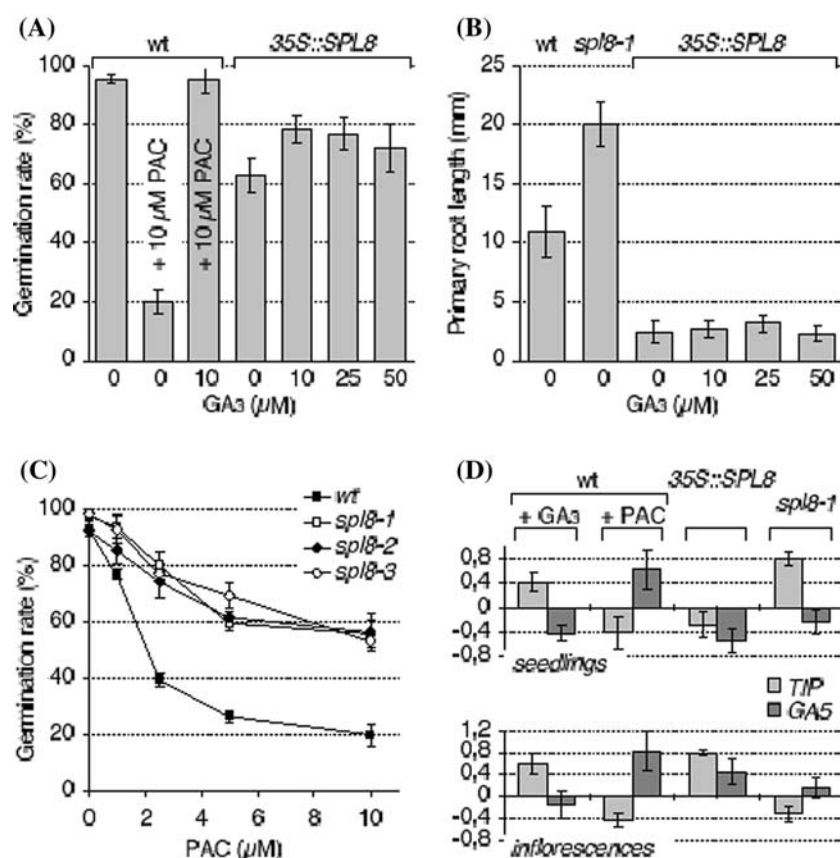


Fig. 5 Alterations in seed germination, root elongation and expression of GA biosynthesis/signalling genes in response to different *SPL8* expression levels. Bioactive GA₃ applied to the medium could neither rescue the low seed germination (**A**) nor the reduced root length (**B**) of *35S::SPL8* transgenics, while *spl8* mutants show an enhanced root length without GA treatment (**B**). (**C**) Germination of *spl8* mutant seeds showed a higher resistance against the GA biosynthesis inhibitor PAC than wild

type. PAC inhibited seed germination of the wild type can be overcome by the application of GA₃ (**A**). (**D**) *GA5* and *γ-TIP* show altered transcript levels in seedlings (top) and inflorescences (bottom) of wild-type treated either with 50 μM GA₃ or 10 μM PAC and *SPL8* gain- and loss-of-function plants. Fold changes in transcript levels are depicted in comparison to non-treated wild type after normalization against endogenous *RAN3* transcript levels

during the seedling stage regarding to seed germination and root elongation.

Expression of GA biosynthetic/signalling components were altered by *SPL8*

Alterations in endogenous GA levels/responses are generally reflected in changed transcript levels of GA biosynthetic/responsive genes. Among the typical marker genes, *GA5* and *γ-TIP* have often been used as indicators for such alterations in Arabidopsis. *GA5* encodes a key GA biosynthetic enzyme GA 20-oxidase and has found to be transcriptionally feedback controlled by GAs (Xu et al. 1999; Meier et al. 2001). *γ-TIP* encodes a tonoplast intrinsic protein whose transcript level is positively regulated by GAs (Cowling et al. 1998).

As *SPL8* gain- and loss-of-function mutants displayed such contrasting effects in response to GA during seedling stage, we tested the possibility that *SPL8* affects the endogenous GA level/response. For that purpose, transcript levels of *GA5* and *γ-TIP* were compared in seedlings at 5 DAG and in inflorescences of wild type, *35S::SPL8*, and *spl8-1*. As shown in Fig. 5D, the transcript levels of both genes were altered by GA/PAC treatment as well as *SPL8* gain- and loss-of-function, indicating that *SPL8* indeed affects the endogenous GA level/response.

Discussion

A requirement for local acting regulators in GA signalling is inherent to the diverse local effects of GA on plant development. Most GA signalling components

identified so far affect a full spectrum of GA regulated developmental processes, suggesting that they act rather early in the GA signalling cascade. Less is known about downstream components acting as local regulators/modulators of GA responses.

The SBP-box gene *SPL8* is required for proper micro- and megasporogenesis in Arabidopsis and loss-of-function mutants display a semi-sterile phenotype (Unte et al. 2003). In this report, we present evidence that *SPL8* likely acts as a local GA signalling regulator/modulator playing distinct roles depending on its spatiotemporal expression.

SPL8 positively modulates GA signalling in the flower

Sterility and reduced stamen filaments are characteristic features of most GA-deficient/insensitive mutants and depending on the plant species, GA-deficiency/insensitivity affects different stages of anther development (Jacobsen and Olszewski 1991; Izhaki et al. 2002; Huang et al. 2003; Cheng et al. 2004). Sterility has also been reported in case of a GA overdose (Colombo and Favret 1996) and due to a constitutive GA response caused either by removing the *SLENDER RICE1* gene function in rice (Ikeda et al. 2001), by removing the function of a negative GA signalling regulator *SPY* in Arabidopsis (Jacobsen and Olszewski 1993), by overexpressing a positive GA signalling regulator *HvG-AMYB* 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, a GA overdose/constitutive response causes anther non-dehiscence in all species studied so far (Colombo and Favret 1996; Murray et al. 2003; our study on *spy-3*).

In addition to abnormal stamen development, *spl8* mutants showed a reduction in the density of the trichomes found on sepals, which is also known to be regulated by GA (Perazza et al. 1998). Both features could not be restored through the application of GA but *spl8* mutants did respond with internode elongation in their inflorescences similar as wild type. Thus *spl8* mutants have either become locally insensitive to GA or the respective floral features altered independent of GA.

Our data on *SPL8* gain-of-function transgenics does support the first possibility. Plants constitutively expressing *SPL8* displayed an increased sepal trichome density and complexity, normal stamen filaments and anther non-dehiscence. We ruled out two well-known possible causes of anther non-dehiscence, disturbed JA and ethylene signalling. In addition to anther non-dehiscence, JA-insensitivity/deficiency results in pollen

inviability, which is not the case for *SPL8* gain-of-function lines. Ethylene is also unlikely to be directly related to the function of *SPL8*. Although ethylene deficiency/insensitivity delays anther dehiscence such that pollen release is not properly synchronized with flower opening, anther structure as such remains unaffected (Rieu et al. 2003). Based on our results, a GA constitutive response best explains the *SPL8* gain-of-function phenotype. That *SPL8* loss- and gain-of-function resemble GA insensitive and constitutive response respectively, suggests that *SPL8* acts as a positive regulator in GA-mediated anther development.

SPL8 negatively modulates GA signalling in the seedling

In addition to its promoting role in reproductive growth, we found *SPL8* to act as a repressor in GA-mediated seed germination and root elongation. GA promotes both developmental processes through counteracting the inhibitory effect of DELLA proteins (Lee et al. 2002; Fu and Harberd 2003; Tyler et al. 2004). GA-deficiency, as observed in *gal-3* mutants, results in germination failure and shortened roots (Koornneef and Van der Veen 1980; Fu and Harberd 2003). In contrast, constitutive GA-response mutants show a more PAC-resistant germination potential and can compensate for the reduced root growth of *gal-3* (Jacobsen and Olszewski 1993; Lee et al. 2002; Fu and Harberd 2003; Tyler et al. 2004). With respect to seed germination and root growth, *SPL8* loss- and gain-of-function mutants behaved like constitutive GA-response mutants and GA-deficient mutants, respectively.

The dual regulatory role of *SPL8* in GA signalling during the seedling stage and anther development was further strengthened by molecular data. Ross (1994) hypothesized that due to an existing negative feedback mechanism, mutants with an increased GA signal transduction would have reduced levels of active GA, whereas elevated GA levels are to be expected in mutants with a decreased signal transduction. This hypothesis has found much experimental support (Cowling et al. 1998). Given that *SPL8* acts as a positive modulator in GA-signalling during anther development, the enhanced GA signal transduction of *35S::SPL8* would result in reduced endogenous GA level, while *spl8* mutants would have an increased endogenous GA level but reduced GA signal responses. The opposite would be expected in seedling stage considering the dual role *SPL8* plays.

γ -*TIP*, a gene transcriptionally activated in response to bioactive GAs (Cowling et al. 1998), was indeed shown to be up-regulated in *spl8* mutants and down-regulated in *35S::SPL8* transgenic seedlings in comparison to wild type. In inflorescences we observed an opposite behaviour. Based on the phenotypic and hormone treatment analysis, these data support the idea that *SPL8* acts as a negative modulator in GA signalling during seedling development. It obviously performed a contrasting role during GA-regulated anther development according to our phenotypic and molecular data.

Overexpression of *SPL8* resulted in elevation of *GA5* transcript levels in inflorescences, while lowering them in seedlings, indicating a reduced and enhanced endogenous GA level, respectively. However, there seems to be a discrepancy regarding to the *GA5* level found in *spl8* mutants. Cowling et al. (1998) demonstrated that dominant GA-insensitive *gai* mutants contained high endogenous GA levels and also 4-fold higher *GA4* transcript levels and suggested a perturbed feedback regulation in these mutants. Similarly, removing *SPL8* function could disturb a GA-dependent feedback regulation on *GA5*.

Conclusion

In conclusion, our data let us to propose that *SPL8* plays a dual role in the local regulation of a subset of GA-mediated developmental processes. *SPL8* positively modulates GA signalling in the flower, whereas negatively in the seedling. Additional studies are needed to determine how *SPL8* is integrated in the implementation of the GA signal. In particular, it will be interesting to find out if GA is the input signal regulating the nuclear import of *SPL8* through post-translational modification, a mechanism suggested by Birkenbihl et al. (2005).

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