SPLAYED: A chromatin remodeler regulating tissue specific gene silencing and developmental timing

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SUMMARY

A number of mechanisms that contribute to epigenetic regulation of gene expression have been described in plants. The concert actions of these mechanisms contribute to proper patterning of gene expression within organs and tissues, therefore insuring their morphology and functions. However, while the general effects of components of the transcriptional (TGS) and post-transcriptional gene silencing (PTGS) pathways have been reported, the impact of individual elements of these pathways on silencing of target genes at a tissue specific level remained to be investigated.

In this thesis, we addressed the question of regulation of tissue specific gene expression using a transgenic GFP reporter line containing the epigenetically controlled endogenous promoter of *APUM9*. Previous studies have revealed that *APUM9* is under complex epigenetic control. Plants of this line exhibited GFP expression only in siliques (therefore, the line was named "silex"), suggesting that the GFP transgene was silenced in the other tissues. To investigate the role of TGS and PTGS factors in silencing the GFP transgene and to identify novel factors that contribute to this process, forward and reverse genetic approaches were used.

To study the impact of different silencing pathways on suppression of the GFP transgene in silex, plants of the reporter line were crossed to mutants defective in components of the TGS (*nrpe1*) and PTGS (*ago1*, *se*, *sgs3*, *dcl4*) pathways. The study, presented in this thesis, revealed that these factors were all required to suppress of GFP expression in different tissues. This indicated that both TGS and PTGS are involved in silencing of the GFP transgene in silex.

In order to identify novel epigenetic factors, contributing to tissue specific silencing of the GFP transgene, a forward genetic mutant screen was performed on the silex reporter line. This thesis reports on mutant alleles of SPLAYED (SYD, *syd-10* and *syd-11*), a chromatin remodeling ATPase of *Arabidopsis* that were recovered in this screen. *syd-10* and *syd-11*, expressed GFP in the vascular tissues of leaves, stems and in inflorescences. SYD has previously been known to be an important regulator of flower organ identity and homeotic gene expression. The findings described in this thesis now indicate that SYD also contributes to silencing of the GFP transgene in parental silex line and suggest a possible wider role of SYD in silencing.

Further study of the *syd* phenotype indicated a possible connection between SYD and the highly conserved micro-RNA miR156 that plays important roles in regulation of juvenile-to-adult and vegetative-to-reproductive developmental phase transitions. The level of miR156 was reported to be affected by external factors, such as temperature and carbohydrate accumulation. However, so far, only a few molecular factors involved in direct transcriptional control of *MIR156* genes have been identified. This thesis provides evidence that SYD takes part in regulation of developmental phase changes by directly modulating transcription of several *MIR156* and *SPL* genes in *Arabidopsis*.

Generally, the results, presented in this thesis allow us to conclude that the tissue specific silencing of the GFP transgene in the silex line is dependent on components of PTGS as well as TGS, and that both systems may act in a complementary manner. Also, silencing of the GFP expression in the vasculature and inflorescences of silex plants is dependent on the SYD chromatin remodeler and possibly mediated by miR156. Moreover, SYD plays a role in direct transcriptional regulation of miR156 suggesting that it acts in the regulation of miR156-dependent and miR156-independent pathways during plant development.

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CHAPTER I GENERAL INTRODUCTION

1.1 Transcriptional and post-transcriptional gene silencing

Living organisms have a constant need to react and adapt to changes within their environment. Moreover, morphology and physiological characteristics of organisms can undergo drastic changes throughout development and maturation. Therefore, highly precise and flexible mechanisms regulating all these processes are required. Expression of genes can be regulated at both, transcriptional and post-transcriptional levels. Transcriptional gene silencing (TGS) involves establishment of DNA methylation and acquisition of repressive chromatin modifications (including covalent modifications of histones and changes of the chromatin structure by remodeling complexes), whereas posttranscriptional gene silencing (PTGS) results in degradation of the mRNA template and/or in translational repression.

PTGS was first described in plants in 1990 and initially called "co-suppression". It was observed that introduction of an additional copy of dihydroflavonol-4-reductase (DFR) or chalcone synthase (CHS) genes, responsible for pigmentation in petunia lead to loss of pigmentation in petals (instead of an expected intensification of the color) due to suppression of both, the endogenous and the transgene loci (Napoli et al., 1990). Since then, multiple examples of PTGS in different species were reported (Lee et al., 1993, see Cogoni and Macino, 2000) for a detailed review).

As a general term, referred to silencing pathways mediated by small RNAs, the term "RNA silencing" was introduced. It is involved in repression of transposons and transgenes (Mlotshwa et al., 2008) and defense against viruses, viroids (Hamilton and Baulcombe, 1999), reviewed in (Vaucheret, 2006; Vazquez and Hohn, 2013). RNA silencing also plays a role in DNA repair in plants, fungi and *Drosophila* and in DNA elimination in protists (Dang et al., 2011; Michalik et al., 2012; Wei et al., 2012b) Bracht, 2012).

For some time these two pathways were regarded as separate, however recent reports indicate that tight connections exist between them (Vazquez and Hohn, 2013; Castel and Martienssen, 2013; Martínez de Alba et al., 2013). For example, RNA silencing can act in regulation of gene expression at transcriptional (TGS) as well as

at post-transcriptional (PTGS) levels. In human HEK293T cells siRNAs that carry a sequence homology with the promoter region of a target gene, are loaded into ARGONAUTE1 (AGO1) and mediate TGS by formation of silent chromatin domains (Kim and Rossi, 2009). And in plants, 23 -26-nt siRNAs derived from microRNA genes have been reported to drive DNA methylation at target loci (as demonstrated for *SPL2,* target of miR156) (Chellappan et al., 2010).

Therefore, dynamic interplay between the TGS pathway implemented by DNA methylation, histone modifications and components of the PTGS machinery is taking place to enable complex regulation of gene expression.

1.2 Epigenetic marks, involved in regulation of gene expression

The presence of phenotypic differences between genetically identical organisms (or even cells within a multicellular organism) lie at the basis of the concept of epigenetics (for "above" genetics) (Bonasio et al., 2010). Currently, it has been defined as ''the study of changes in gene function that are mitotically and/or meiotically heritable and that do not entail a change in DNA sequence'' (Wu, 2001). The described epigenetic modifications comprise DNA methylation, histone modifications and nucleosome localization. Some of these marks were shown to play roles in genome stability and plant development (Fujimoto et al., 2006; Martin et al., 2009; Shiba et al., 2006; Pfluger and Wagner, 2007; Wagner, 2003). However, as by current definition an epigenetic mark should be heritable, it is a matter of a dispute, whether histone modifications and nucleosome localization can be considered truly epigenetic (discussed later in more detail).

DNA methylation

DNA methylation - adding of a methyl group to the 5th carbon atom of the pyrimidine ring of a cytosine base (5-methylcytosine or 5mC) - is an evolutionary conserved epigenetic mark, involved in gene silencing in eukaryotes (Bird, 1992; Furner and Matzke, 2010; Keshet et al., 1986). In mammals DNA methylation occurs

predominantly at cytosines within the symmetric CG sequence context (Law and Jacobsen, 2010), whereas in *Arabidopsis* DNA methylation has been detected in all three cytosine contexts: CG, CHG and CHH (where H is A, C, or T). Whole genome bisulfite sequencing studies reported that about 55% of DNA methylation in Arabidopsis resides in the CG context, whereas the remaining 45% are equally distributed between methylation in CHG and CHH contexts (Cokus et al., 2008; Lister et al., 2008)

In both, animals and plants DNA methylation plays a major role in silencing transposable elements (TEs) (Yoder et al., 1997; Reinders et al., 2009; Teixeira and Colot, 2010; Tsukahara et al., 2009). TEs are highly abundant in eukaryotic genomes and particularly in plant genomes (Cridland et al., 2013; Rebollo et al., 2012; Fedoroff, 2000). Generally, TEs can be divided into two classes: retrotransposons (class I) and DNA transposons (class II). Transposition of Class I TEs requires a reverse transcription step of an RNA intermediate, resulting in an increase in copy number ("copy-and-paste" mechanism). Translocation of Class II TEs takes place through the "cut-and-paste" mechanism, therefore the copy number remains stable (Wicker et al., 2007). Due to the threat new TE insertions pose to genome integrity, their expression is repressed. In *Arabidopsis*, DNA methylation density correlates with TE density and other repetitive sequences within pericentromeric regions (Zilberman et al., 2006; Cokus et al., 2008).

DNA methylation targeted at TEs can also affect expression of nearby endogenous genes (Ahmed et al., 2011). In plants, a number of genes involved in establishment of self-incompatibility, regulation of flowering and sex determination, are under epigenetic control due to DNA methylation spreading from TEs located in their promoter region (Fujimoto et al., 2006; Kinoshita et al., 2007; Martin et al., 2009; Shiba et al., 2006).

DNA methylation at TEs takes place at both CG and non-CG contexts. (Zilberman and Henikoff, 2007). In addition to DNA methylation at TEs and repeats, recent studies revealed the presence of DNA methylation in the bodies of transcribed genes. DNA methylation in expressed genes is limited to the CG context (Cokus et al., 2008; Lister et al., 2008). While the exact functions of DNA methylation in gene bodies is not well understood yet, it was suggested that such methylation may block transcription initiation from cryptic promoters located within transcribed regions

(Simmen, 1999; Zilberman and Henikoff, 2007).

Establishment, maintenance and removal of DNA methylation

The mechanisms involved in establishment and maintenance of DNA methylation are now well described. Several factors involved in these processes have been identified (Table 1.1). DNA methyltransferase is an enzyme, which uses ATP and S-adenosyl methionine (SAM, the donor of methyl group) to methylate cytosine residues. The first DNA methyltransferase to be reported was the mouse DNA METHYLTRANSFERASE1 (DNMT1) (Gaudet et al., 2004; Gaudet, 1998). Due to its higher affinity for hemi-methylated than for unmethylated CGs and being a component of the DNA replication complex, it is considered to play role in maintenance of DNA methylation during DNA replication (Vertino et al., 2002). During DNA replication in *Arabidopsis*, METHYLTRANSFERASE1 (MET1), a homolog of the mouse DNMT1 protein, is important for maintaining the DNA methylation in CG context (Finnegan et al., 1996; Ronemus et al., 1996). Mutants of MET1 exhibit delayed transition from vegetative to reproductive phase as well as delayed flowering. The developmental phenotype of *met1* becomes more extreme as the mutants are inbred, possibily due to the activation of TEs (Kankel et al., 2003; Ronemus et al., 1996).

DNA methylation in CHG context is mediated by the plant-specific CHROMOMETHYLASE 3 (CMT3) (Lindroth et al., 2001). CMT3 has been demonstrated to play a role in maintenance of CHG DNA methylation at transposon-related sequences (Tompa et al., 2002).

Indicating the contribution of both MET1 and CMT3 to silencing of TEs, the double mutant *met1cmt3* displays significantly stronger effect on reactivation of transposons than either of single mutants (Kato et al., 2003).

Establishment of CHH DNA methylation is thought to be catalysed by DOMAINS REARRANGED METHYLTRANSFERASE 1, 2 (DRM1 and DRM2), homologues of the mammalian *de novo* DNA methyltransferases Dnmt3a and Dnmt3b (Cao et al., 2000; Okano et al., 1999). Plants deficient in DRM1 have been reported to exhibit a delay in flowering under both, long and short day conditions. As

the late flowering phenotype can be restored by vernalization treatment, suggests that DRM1 is associated with the autonomous flowering pathway in *Arabidopsis* (Zhu et al., 2005).

DNA methylation in asymmetric CHH context cannot be maintained from a hemimethylated status, therefore it needs to be reestablished after each round of cell division (*de novo* DNA methylation). Reestablishment of CHH DNA methylation is guided by siRNAs through the RNA directed DNA methylation pathway (RdDM) (Matzke, M. A., & Mosher, R. A., 2014; Simon and Meyers, 2010). CHH methylation was detected to be targeted to both, endogenous repeat elements and transgenes (Matzke et al., 1989). Notably, in the *drm1drm2cmt3* triple mutant CHH methylation is not completely obliterated, which indicates that other yet unknown DNA methyltransferases may contribute to DNA methylation at this context (Henderson and Jacobsen; Lister et al., 2008).

In addition to DNA methyltransferases, several other proteins and protein-complexes are enrolled in maintenance and *de novo* DNA methylation mechanisms. The DECREASE IN DNA METHYLATION 1 (DDM1) SWI2/SNF2 chromatin remodeler ATPase has been shown to play a role in maintenance of the DNA methylation over repeat elements (Richards et al., 1999; Teixeira et al., 2009). *ddm1* plants do not display strong developmental abnormalities. However, similar to *met1* the propagation of self-pollinated *ddm1* lines results in severe abnormal morphological phenotypes due to reactivation of TEs (Kakutani et al., 1996). The histone methyltransferase SUPPRESSOR OF VARIEGATION 3-9 HOMOLOGUE 4 (SUVH4), (also known as KRYPTONITE (KYP)), is involved in histone H3K9 dimethylation (H3K9me2) (Jackson et al., 2002) and is required for the maintenance of CNG methylation together with CMT3. It has been suggested that other histone methyltransferases might also be involved in this process (Ebbs, 2006; Law and Jacobsen, 2010).

Plant factor	Function	Homologues in other species
METHYLTRANSFERASE 1 (MET1/DDM2)	Maintenance of CG methylation	Mouse - Dnmt1 (DNMT)
DECREASED IN DNA METHYLATION1 (DDM1)	DNA methylation over repeat elements (mainly in CG and CHG context)	Mouse - Lsh (SWI/SNF ATPase)
DOMAINS REARRANGED METHYLTRANSFERASEs (DRM1, DRM2)	Establishment of CHH methylation	Mammals - Dnmt3 (DNMT)
CHROMOMETHYLASE3 (CMT3)	Maintenance of CHG DNA methylation	Specific to the plant kingdom
REPRESSOR OF SILENCING1 (ROS1)	DNA demethylation	HhH-GPD (helix-hairpin-helix followed by a Gly-Pro rich loop and a conserved Asp) superfamily DNA glycosylases ROS1/DME proteins appear to be plant specific
DEMETER (DME)	DNA demethylation demethylation of repeat elements. May be involved in imprinting in the endosperm	
DEMETER-LIKE2 (DML2)	DNA demethylation	
DEMETER-LIKE3 (DML3)	DNA demethylation	

Table 1.1 Components, involved in DNA methylation in plants and their homologues in other species (He X-J, et al., 2011; Meyer P 2011; Wagner D, 2003)

DNA demethylation can occur passively, due to loss of methylation maintenance and *de novo* DNA methylation activity during DNA replication. In this case DNA methylation will not be reestablished on the newly synthetized DNA and therefore DNA methylation will be lost with every replication round. Also, DNA demethylation can be achieved via active elimination of methylated cytosines by DNA glycosylases (Zhu, 2009). Four DNA glycosylases have so far been identified in *Arabidopsis*: REPRESSOR OF SILEMCING1 (ROS1), DEMETER (DME), DEMETER-LIKE2 (DML2) and DML3 (Penterman et al., 2007; Gehring et al., 2006; Ortega-Galisteo et al., 2008).

Expression of DME was only detected in the central cell during the late stages of female gametogenesis. DME was shown to be involved in genomic imprinting in the endosperm (a allele-specific expression of a gene dependent on its parent-of-origin) (Messing and Grossniklaus, 1999; Köhler and Weinhofer-Molisch, 2009; Gehring et al., 2004; Bauer and Fischer, 2011; Kinoshita, 2004; Xiao et al., 2003). On the other hand, ROS1 and DML2, 3 were reported to be ubiquitously expressed

(Ortega-Galisteo et al., 2008)

RNA-directed DNA methylation

Initially, the process of RNA directed DNA methylation (RdDM) was described in viroid infected tobacco plants by (Wassenegger et al., 1994). DNA methylation was restricted to inserted viroid cDNA sequences and was shown to be triggered by autonomous viroid RNA-RNA replication (Wassenegger et al., 1994). Later, silencing of a hygromycin phosphotransferase (hpt) transgene promoter by DNA methylation was shown to be induced by sequence homology (Park et al., 1996).

RdDM is involved in transposon silencing, genome stability and it also plays a role in imprinting and in regulating expression of developmental genes (Mathieu and Bender, 2004; Takeda and Paszkowski, 2006; Bauer and Fischer, 2011; Teixeira and Colot, 2010; Mirouze et al., 2009; Ito, H et al., 2011). *FLOWERING WAGENINGEN* (*FWA*) is one of the genes in *Arabidopsis* reported to be under regulation by RdDM and active DNA demethylation. This developmental gene encodes for a homeobox transcription factor. Silencing of *FWA* is induced by the presence of transposon-associated tandem repeats in its promoter. These tandem repeats trigger the production of siRNAs resulting in the accumulation of DNA methylation within the *FWA* promoter thus silencing it (Kinoshita et al., 2007; Chan et al., 2006b). *FWA* has been shown to be imprinted and its expression is restricted to the maternal genome of the endosperm. This is achieved through removal of the DNA methylation from the maternal allele in the central cell of the female gametophyte by DME (Matzke et al., 2007).

The process of RdDM in plants requires the activity of the plant specific RNA polymerases Pol IV and PolV (Herr et al., 2005; Pikaard et al., 2008; Mosher et al., 2008; Haag and Pikaard, 2011). Characterisation of the structure of these enzymes revealed that both RNA polymerases consist of 12 subunits, some of which are in common between Pol IV and Pol V and some are also present in Pol II. The largest subunits of Pol IV (NRPD1) and Pol V (NRPE1) are unique and second largest subunit NRPD2/NRPE2 is shared between these two RNA polymerases (Ream et al., 2009; Tucker et al., 2010).

The current model for RdDM suggests that Pol IV is recruited to target loci to produce a single-stranded RNA transcript (Herr et al., 2005; Mosher et al., 2008). Then these template transcripts are converted into a double-stranded RNA (dsRNA) by RNA-DEPENDENT RNA POLYMERASE 2 (RDR2) which is subsequently cleaved by DICER-LIKE3 (DCL3) into 24-nucleotides siRNAs (Daxinger et al., 2009). These siRNAs are loaded into Argonaute proteins (AGO4, 6 or 9) (Qi et al., 2006; Hutvagner and Simard, 2008; Martínez de Alba et al., 2013) forming a RNA-induced silencing complex (RISC) (Figure 1.1). This complex interacts with PolV which produces non-coding transcripts from silent loci (Mosher et al., 2008; (Pikaard et al., 2008). Interaction of RISC with nascent Pol V transcripts recruits DOMAINS REARRANGED METHYLASE 2 (DRM2) to target loci to initiate subsequent DNA methylation in the region of siRNA-DNA homology (Martínez de Alba et al., 2013; Daxinger et al., 2009; Teixeira and Colot, 2010) (Figure 1.1).

Thus, the establishment of RdDM is mediated by Pol IV-dependent siRNAs that guide Pol V to target DNA methylation at homologous loci (Chinnusamy and Zhu, 2009; Haag and Pikaard, 2011; Wassenegger and Krczal, 2006) (Figure 1.1).

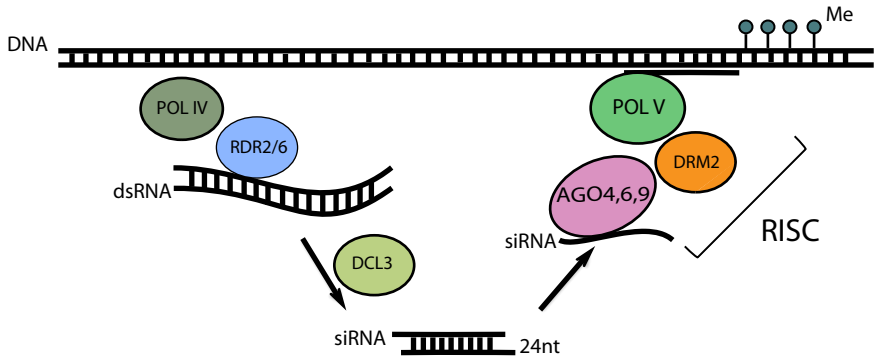


Figure 1.1 The RNA-directed DNA methylation pathway

The putative RNA polymerase Pol IV mediates the production of RNA transcripts. These transcripts are further converted to dsRNA by RDR2 and cleaved to 24-nt sRNAs by DCL3. Mature sRNAs are loaded into AGO4 and AGO6 (and maybe AGO9) to mediate the recruitment of RNA polymerase Pol V to complementary loci and target the establishment of *de novo* DNA methylation.

Histone modifications

The nucleosome is the basic subunit of chromatin. It consists of an octamer of four core histone proteins H2A, H2B, H3 and H4 (with two copies of each) and the DNA, which is wrapped around them. This structure is highly conserved in both, plants and animals. N-terminal tails of histones can be covalently modified at different positions (predominantly lysine and arginine residues) by various marks, such as methylation, acetylation, phosphorylation, biotinylation and ubiquitination (Feng and Jacobsen, 2011). The functions of these marks depend on the position, the type of histone and as well as on the organism. These modifications can provide binding sites for both activating and repressing transcriptional regulators thus, influencing the transcription of genes (Latham and Dent, 2007).

The active state of genes is supported by protein complexes of the Trithorax group (TrxG) supporting the permissive chromatin marks, whereas maintenance of silenced state of genes by establishment of repressive chromatin marks is carried out by members of Polycomb group (PcG) (Guitton and Berger, 2005).

In plants, as well as in animals, histone H3K4 mono/di/tri-methylation (H3K4me1, H3K4me2, H3K4me3) are known as permissive chromatin marks, whereas H3K9 di-methylation (H3K9me2) and histone H3K27 tri-methylation (H3K27me3) are repressive marks (Berr et al., 2011; Bernstein et al., 2006; Jenuwein and Allis, 2001).

H3K4 methylation is thought to mark active chromatin. In *Arabidopsis*, H3K4me3 was found to be enriched at actively transcribed regions. High levels of H3K4me3 were detected at the Transcription Start Site (TSS) of active genes, extending to the first 300bp of the transcribed DNA region (Zhang et al., 2009). H3K4me1, H3K4me2 were also detected within transcribed regions, however their function in gene activation has not yet been confirmed (Zhang et al., 2009).

The H3K4me3 is deposited by proteins of the Trithorax group (TrxG). In *Arabidopsis,* this function is carried out by SET domain (Suppressor of variegation, *E*nhancer of zeste and *T*rithorax, responsible for methyltransferase activity) containing proteins, homologous to components of TrxG from other species. Among them are the ARABIDOPSIS HOMOLOG OF TRITHORAX1,2 (ATX1,2) and the

ARABIDOPSIS TRITHORAX-RELATED 3, 7 (ATXR3, also known as SDG2, ATRX7) (Baumbusch, 2001; Saleh et al., 2008; Tamada et al., 2009; Valencia-Morales et al., 2012; Guo et al., 2010).

Generally, H3K9me3 is considered to be a heterochromatic mark, however in *Arabidopsis* it is localized in euchromatin and can be detected at transcriptionally active genes (Fuchs et al., 2006). At the same time H3K9me2 was found to be a hallmark of constitutive heterochromatin in *Arabidopsis* (Fuchs et al., 2006; (Nakayama, 2001; Ramirez-Parra and Gutierrez, 2007). H3K9me2 contributes to establishment of the DNA methylation at pericentromeric regions and over TEs (Bernatavichute et al., 2008; Furner and Matzke, 2010). Propagation of H3K9me2 is performed by histone methyltransferases, including SUVH4/KYP, SUVH5 and SUVH6 (Jackson et al., 2004; Ebbs et al., 2005; Ebbs, 2006).

H3K27me3, deposited by Polycomb group (PcG) proteins is associated with repressed genes. In animals, transcriptional repression by Polycomb group proteins is mediated by the action of two complexes: Polycomb Repressive Complex 1 and 2 (PRC1 and PRC2). The PRC2 complex is structurally and functionally conserved between the plant and animal kingdoms, and components of PRC2 have been characterized in *Arabidopsis* (Guitton and Berger, 2005). In contrast, PRC1 homologs diverge between species. PRC1 function was long thought to be absent in *Arabidopsis*, as no obvious homologue of the animal PRC1 subunit could be identified (Whitcomb et al., 2007). Only recently, plant-specific proteins carrying PRC1 function, EMBRYONIC FLOWER1 (EMF1) and LIKE HETEROCHROMATIN PROTEIN1 (LHP1), have been discovered (Calonje et al., 2008; Hennig and Derkacheva, 2009). Further identification of homologs of other Polycomb group (PcG) components in plants suggested that the general mechanism of Polycomb repression is similar between these species (Calonje, 2013; Hennig and Derkacheva, 2009). Interestingly, it has been demonstrated that the H3K27me3-modified regions in *Arabidopsis* generally are significantly shorter than those in *Drosophila* and mammals (Zhang et al., 2007).

In *Arabidopsis*, H3K27 tri-methylation plays roles in epigenetic silencing of a number of known developmental genes, such as the flower timing gene *FLOWERING LOCUS C (FLC)*, floral organ patterning gene *AGAMOUS (AG), PHERES1 (PHE1)* (Lopez-Vernaza et al., 2012).

There is an ongoing discussion whether histone modifications can be considered as true epigenetic marks. By the accepted definition of "epigenetics", such marks should be inheritable through mitosis or meiosis (Allis et al., 2006; Bonasio et al., 2010). Among known histone modifications, the H3K4me3 and H3K27me3, established by TrxG and PcG proteins (respectively) were considered most likely to be the true epigenetic marks (Hansen et al., 2008; Molitor and Shen, 2013; Ng and Gurdon, 2008; Saleh et al., 2007).

To check this assumption it was tested if H3K4me3 and H3K27me3 can be detected on histones shortly after the DNA replication during the Drosophila development (Petruk et al., 2012). Based on the results of this study, it was suggested that the role of true epigenetic marks may be played by the TrxG and PcG proteins, rather than the histone modifications themselves. The TrxG and PcG proteins establish stable interactions with their target DNA sequences and, possibly, with components of DNA polymerase complex during replication. While the methylated histones are being exchanged with unmethylated once, continuous presence of TrxG and PcG proteins may guide further reestablishment of corresponding histone modifications (Petruk et al., 2012).

1.3 Chromatin remodeling

General characterization of chromatin remodeling ATPases

SWI/SNF ATPases bare their name from the Snf2 protein, which was first described to be required for anaerobic fermentation of sucrose (SWItch/Sucrose Non-Fermentable) in *Saccharomyces cerevisiae* (Abrams et al., 1986). It was later shown to play an important role in other molecular processes, such as regulation of transcription and chromatin stability (Becker and Hörz, 2002; Euskirchen et al., 2012; Carlson and Botstein, 1982; Vries, 2005).

Close orthologs of *S. cerevisiae* Snf2p have been found in other model organisms (Cairns et al., 1996; H Chiba, 1994; Euskirchen et al., 2012; Muchardt and Yaniv, 1999; Tamkun et al., 1992; Wang et al., 1996). Based on sequence alignments of the helicase-like region, 24 distinct subfamilies of the Snf2 family proteins have been distinguished (Eberharter, 2004; Flaus et al., 2006; Lusser and Kadonaga, 2003). The names of these subfamilies have been assigned by the name of the archetypal member, such as *S. cerevisiae* Snf2p (SWI/SNF subfamily), *Drosophila melanogaster* Iswi (ISWI subfamily) and the *Mus musculus* Chd1 (CHD subfamily) (Flaus et al., 2006). Consequently, the *S. cerevisiae* Snf2p gave name to the specific SWI/SNF subfamily as well as to general Snf2 family (Flaus et al., 2006; Lusser and Kadonaga, 2003). It is thought, that eukaryotic organisms contain proteins from at least six major subfamilies of Snf2 chromatin remodelers: SWI/SNF, ISWI, NURD/Mi-2/CHD, INO80 and SWR1 RAD54 (Knizewski et al., 2008; Saha et al., 2006).

The recent studies were largely focused on SWI/SNF subfamily members, which play roles in chromatin remodeling. It has been reported that SWI/SNF ATPases can trigger sliding of the nucleosome (Becker and Hörz, 2002; Kassabov et al., 2003), alterations of histone DNA contacts (Narlikar et al., 2001), partial or complete removal of the histone octamer components (Bao and Shen, 2007; Bruno et al., 2003). Mutations in subunits of SWI/SNF complex have been linked to malignant transformation (Versteege et al., 1998). Members of this subfamily have been reported to interact with other regulatory proteins such as histone deacetylases, histone methyl transferases and histone chaperones (Sif et al., 2001; Zhang et al., 2000; Moshkin et al., 2002; Xu, 2004) and Pol II (Wilson et al., 1996).

More than 40 SWI/SNF proteins have been identified in *Arabidopsis* (www.chromdb.org). A further classification of the SWI/SNF subfamily proteins is available at http://www.snf2.net/ (Flaus et al., 2006). Although active chromatin remodeling complexes have not been purified in *Arabidopsis* yet, a number of SWI/SNF subfamily proteins were reported as subunits of such complexes (Jerzmanowski, 2007; Saha et al., 2006). Among them are two large SWI/SNF ATP-ases SPLAYED (SYD) and BRAHMA (BRM) (Bezhani et al., 2007; Farrona, 2004; Wagner and Meyerowitz, 2002; Wagner, 2003). A number of smaller SWI/SNF proteins, such as CHR12 and CHR23 (Mlynárová et al., 2007; Knizewski et al., 2008; Sang et al., 2012), SWP73A, SWP73B (Sarnowski et al., 2005; Jerzmanowski, 2007) and SWI3A - D (Sarnowski et al., 2005) were identified as subunits of plant chromatin remodeling complexes, based on sequence similarities with subunits of corresponding complexes in metazoa. It has been suggested that combinations of these subunits might allow formation of complexes with different specificities (Jerzmanowski, 2007; Reyes, 2014; the Chromatin Database, www.chromdb.org)

Functioning of chromatin remodeling complex

Although recent studies reveiled some genetic and physical interractions between different subunits of plant chromatin remodeling complexes, the precise mechanism of chromatin remodeling in plants have not been well studied yet (Vercruyssen et al., 2014). Considering the structural similarities of the SWI/SNF subunits, the DNA translocation in plants may be achieved by a mechanism similar to the one suggested for the canonical yeast SWI/SNF remodeler (Saha et al., 2006) (Figure 1.2). Following this model, the ATPase subunit of chromatin remodelers can be divided into torsion and tracking subdomains. Series of ATP-dependent movements of these subdomains break histone-DNA interactions and enable directional sliding of DNA.

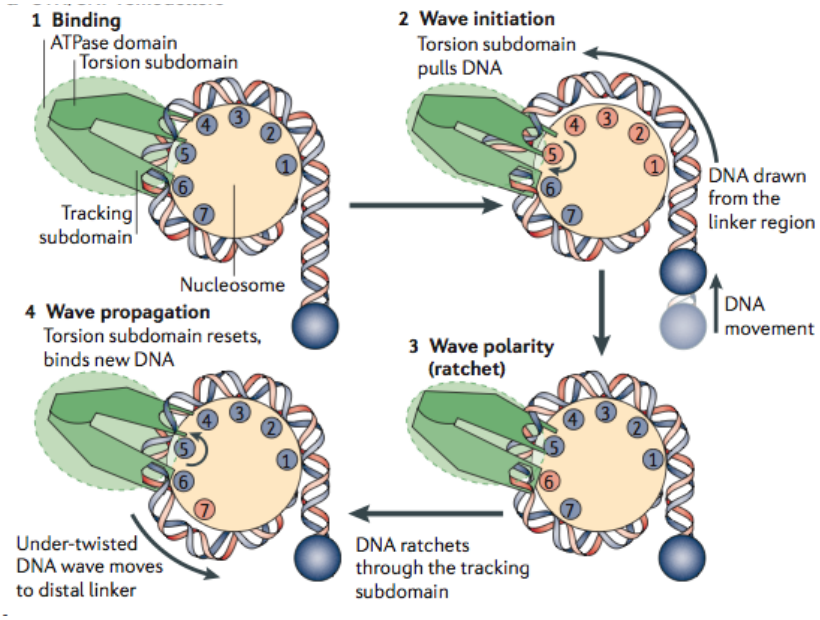


Figure 1.2 The wave-ratchet-wave model for DNA translocation on histones

The SWI/SNF remodeler ATPase protein contains torsion and tracking subdomains. These subdomains go through series of movements, breaking the contacts between DNA and histones within nucleosomes. Blue numbered circles represent the histones with intact and red with broken interactions with the DNA. Four main steps can be distinguished in the process of chromatin remodeling: 1 – The ATPase domain of the SWI/SNF remodeler binds to the nucleosome core; 2 – ATP-dependent conformational change within the tracking subdomain leads to pulling of DNA in the direction from the linker region into the nucleosome, creating the DNA wave; 3 – The tracking subdomain releases DNA, allowing the wave to pass in 3' – 5' direction; 4 – The torsion subdomain of chromatin remodeler reestablishes its connections with DNA at a new position and initiated DNA wave spreads downstream, breaking histone-DNA contacts. The model was adapted from (Saha et al., 2006).

The process of chromatin remodeling consists of four main stages. First, the chromatin remodeller binds to nucleosome core with ATPase domain approximately 2 DNA turns from the nucleosome dyad (Figure 1.2, 1). Next, the ATP - dependent conformational change within the tracking subdomain triggers the dislocation of DNA, pulled by the torsion subdomain, from the linker region in the direction towards the centre of the nucleosome (Figure 1.2, 2). This creates a wave of DNA between the subdomains. Further, the DNA wave passes through the tracking subdomain (Figure 1.2, 3) in 3′→5′ direction and propagates along the nucleosome. The propagation of

the DNA wave leads to distortion of the histone-DNA interactions at the leading edge. Completing the remodeling cycle, these interactions are then reestablished at lagging edge of the nucleosome (Figure 1.2). Then another round of the DNA translocation can take place (Saha et al., 2005). Consistent with this model, it was demonstrated that SWI/SNF activity results in nucleosome movement of 50 bp (Zofall et al., 2006).

SPLAYED a chromatin remodeling ATPase of *Arabidopsis*

SPLAYED (SYD) is one of the core SWI/SNF ATPases identified in *Arabidopsis* (Flaus et al., 2006). It is closely related to BRAHMA (BRM), another member of the same protein family (Farrona, 2004; Bezhani et al., 2007; Knizewski et al., 2008). The core SWI/SNF ATPases have common structure at the N-terminus and contain the Helicase-SANT–associated domain (HSA, also called "Domain 2") and the ATPase region that comprises the SNF2 and helicase domains, responsible for the enzymatic activity (Jerzmanowski, 2007). It was suggested that the N-terminal fragment of SYD, homologous to the corresponding regions of other core SWI/SNF ATPases may also be responsible for interactions with the SWI3- and SNF5-type subunits of the chromatin remodeling complex (Jerzmanowski, 2007; Treich et al., 1995; Tsukiyama and Wu, 1997). Recent studies have reported that the HSA domain is required to mediate such interactions in mammalian SWI/SNF complexes (Trotter et al., 2008). Also, by Szerlong et al., 2008, it was demonstrated that HSA is involved in regulation of chromatin remodeling activity by binding the nuclear actin-related proteins (ARPs).

The C-terminal sequences of the core SWI/SNF ATPases of *Arabidopsis* are different. Unlike much smaller proteins such as CHR12 and CHR23 both, SYD and BRM have large and acidic C-terminal region that contains AT-hook motifs (Jerzmanowski, 2007; Su et al., 2006). These small protein motifs, required for DNA-binding activity, were first identified in the mammalian high mobility group proteins (HMGI/Y) chromosomal proteins that bind to the narrow minor groove of AT-rich DNA, and which are involved in regulation of gene expression (Aravind and Landsman, 1998; Reeves and Nissen, 1990; Reeves and Beckerbauer, 2001).

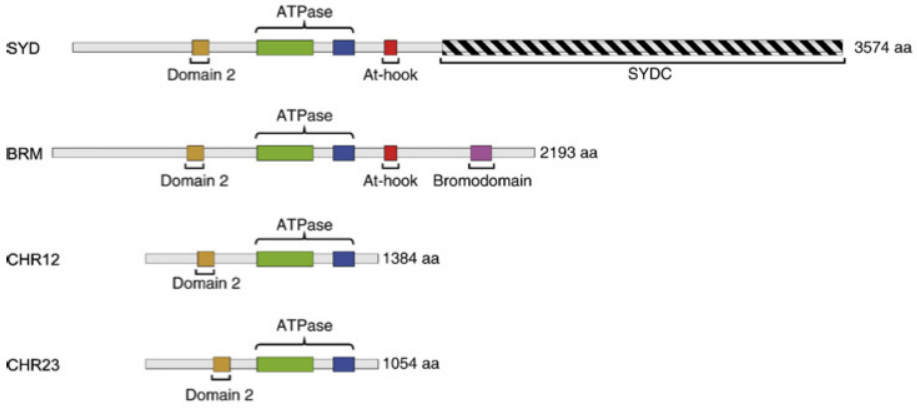


Figure 1.3 Size comparison and domain organization of four *Arabidopsis* SWI/SNF ATPases

The N-terminal part of the four SWI/SNF ATPases contain a Domain 2 motif, which is highly conserved and present in yeast and animal SWI/SNF ATPases (shown in yellow) and the ATPase region, that includes the SNF2 (shown in green) and helicase (displayed in blue) domains. In the C-terminal region of both SYD and BRM there are AT-hook motifs (marked in red), which are typically found in HMG I/Y DNA-binding domains. Of the four SWI/SNF ATPases of *Arabidopsis* that were identified as core subunits of plant chromatin remodeling complexes only BRM has a C-terminal bromodomain (shown in purple). The large C-terminal part of SYD (SYDC) can be cleaved *in vivo*. At the same time the two smaller ATPases, CHR12 and CHR23, lack distinctive C-terminal domains (Jerzmanowski, 2007).

While C-terminal region of BRM was shown to contain a bromodomain (110 amino acids motif, capable of binding acetylated histones) (Jerzmanowski, 2007; Kasten et al., 2004), the large (220 kDa) C-terminal part of SYD after the AT-hooks (SYDC, see Figure 1.3) contains two repeat regions (Wagner and Meyerowitz, 2002). It was suggested that this region might have a plant-specific regulatory functions (Wagner and Meyerowitz, 2002). However, it was reported that the C-terminal part of SYD can be cleaved off *in vivo*, and that the N-terminal fragment including the AT-hook motif is sufficient for securing the biological function (Su et al., 2006). Therefore during development, SYD can be present in two forms: full size (about 400 kDa) and a truncated polypeptide (close to 200 kDa), containing the N-terminal fragment including the AT-hook motif. Full-sized SYD has been shown to be the

predominant form in early development while truncated SYD is more abundant in adult plants (Su et al., 2006; Jerzmanowski, 2007).

Mutants of both *SYD* and *BRM* exhibit strong developmental phenotypes, variable floral homeotic defects (Bezhani et al., 2007; Hurtado et al., 2006; Kwon et al., 2006; Wagner and Meyerowitz, 2002). Although the single null mutants of *brm* and *syd* are not embryo lethal (Farrona, 2004, Hurtado et al., 2006, Wagner and Meyerowitz, 2002), development of a double *brm/syd* mutant is arrested at the heart stage of the embryo (Bezhani et al., 2007). The essential roles of both proteins in early development have been reported, demonstrating their shared and unique functions. At the same time, differences between phenotypes of adult *brm* and *syd* mutant plants suggest that BRM and SYD, may control different molecular events (Kwon et al., 2006; Bezhani et al., 2007).

Emphasizing the important functions of SYD in plant development, the role of SYD in control of *WUSCHEL* (*WUS*, regulator of SAM maintenance) and *AGAMOUS* (*AG*, regulator of floral organ identity) transcription were revealed (Kwon et al., 2005; Wu et al., 2012). Also, SYD was shown to be required for selective pathogen resistance by regulation of transcription of jasmonic acid and ethylene-dependent genes (Walley et al., 2008).

1.4 Plant miRNA and ta-siRNA signaling pathways

Since being first described by (Lee et al., 1993), small non-coding RNAs (snRNAs) have been demonstrated to play important roles in regulation of developmental processes in both, plants and animals (Reinhart, 2002).

Depending on their origin, biogenesis pathway, final length, and mode of action, snRNAs are divided into different groups, such as miRNAs, ta-si RNAs, and long siRNAs (Pulido and Laufs, 2010; Simon and Meyers, 2010; Vazquez and Hohn, 2013).

For a long time, mRNA cleavage was considered to be the main mode of action for plant miRNAs. Regulation of APETALA2 transcription factor by miR172 via translational repression was considered to be an exception (Chen, 2004). However currently there are indications of that miRNA – triggered translational repression may play a role in regulation of multiple genes (Gandikota et al., 2007; Brodersen et al.,

2008). Also, snRNAs can mediate the establishment of DNA methylation at targeted loci via the previously discussed RdDM pathway (Simon and Meyers, 2010). The factors that determine, which of these mechanisms is to act in every particular case have not yet been studied in detail. It was suggested that a high sequence complementarity between the miRNA and the target mRNA is required for triggering cleavage of targeted transcripts. If the sequence complementarity is imperfect, translational inhibition is more likely to take place (Ehrenreich and Purugganan, 2008).

Biogenesis of plant micro-RNAs

miRNAs are produced from larger RNA precursor transcripts that contain a self-complementary structure, which allows the formation of a hairpin. Such precursor transcripts are later recognised by a protein complex, which includes the type III ribo-endonuclease DICER-LIKE1 (DCL1) (Tang, 2005; Kurihara and Watanabe, 2004; Kurihara, 2005), DAWDLE (DDL) (Yu et al., 2008), HYPONASTIC LEAVES1 (HYL1) (Kurihara, 2005), and SERRATE (SE) (Prigge and Wagner, 2001; Grigg et al., 2005; Lobbes et al., 2006). DDL most likely facilitates RNA precursor recognition (Yu et al., 2008). The double-stranded RNA (dsRNA) binding protein HYL1 and SE facilitate the cleavage of the precursor transcript by DCL1 (Kurihara, 2005; Lobbes et al., 2006). As a result of synchronized action of the components of this complex, miRNA precursors are being processed into 20 - 22 nt miRNA–miRNA* duplexes (the miRNA strand of the duplex is selected to enter into the RNA induced silencing complex (RISC), and the miRNA*, or passenger strand is most often degraded). Further, the 3' end of miRNAs is methylated by the HUA ENHANCER 1 (HEN1) methyltransferase to protect it from degradation (Park et al., 2002) (Figure 1.4).

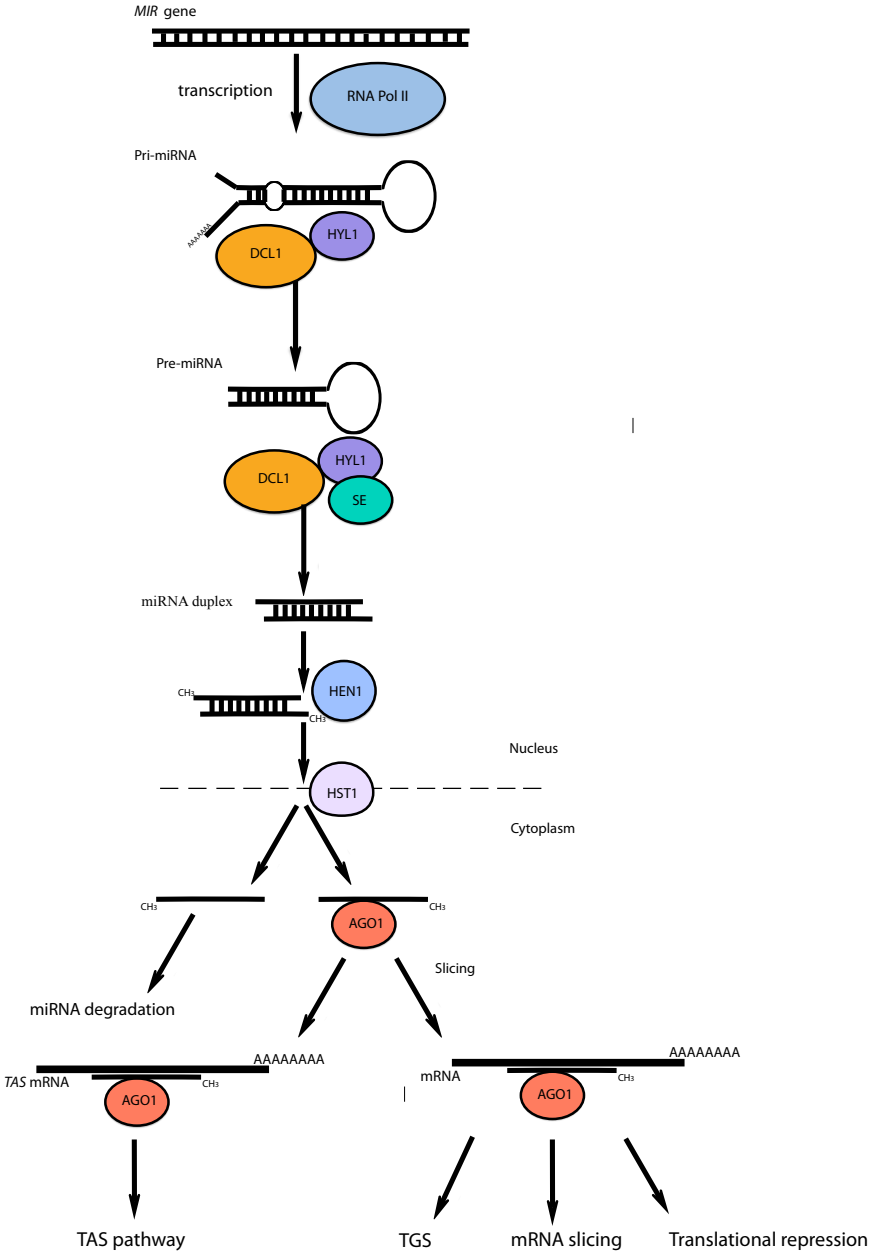


Figure 1.4 Pathway of micro-RNA biogenesis

miRNA precursor molecules (pri-miRNA) are transcribed from dedicated genes. The pri-miRNA transcripts contain self-complementary sequences that allow formation of a hairpin structure. Pri-miRNA transcripts are recognized by a complex of factors, including DCL1, HYL1 and SE that stabilize and cleave them leading to formation of a miRNA douplex (miRNA–miRNA*). The 3' end of the miRNA is methylated by HEN1 methyltransferase and exported to cytoplasm by HST. The mature miRNA is then recognized by AGO1 and included into RISC to mediate silencing of a target gene by mRNA degradation or translational repression.

The mature miRNA duplex is exported to cytoplasm by HASTY (HST) (Park et al., 2005). In the cytoplasm, the miRNA duplex is separated and then single-stranded miRNAs enter RISC (Tang, 2005). miRNAs are predominantly associated with AGO1 (Baumberger and Baulcombe, 2005; Hutvagner and Simard, 2008). This complex guides cleavage of the target mRNAs and/or translational repression (Brodersen and Voinnet, 2009; Hu and Coller, 2012; Llave et al., 2002; Wightman et al., 1993; Mallory and Vaucheret, 2010) (Figure 1.4). Generally, miRNAs contribute to regulation of spatiotemporal gene expression patterns, also playing a buffering role, preventing minor changes in target expression levels (Pulido and Laufs, 2010).

Role of miRNAs in plant development

miRNAs have been demonstrated to act in regulation of plant development (Pulido and Laufs, 2010; Willmann et al., 2011; Yamaguchi and Abe, 2012). Their vital importance in this process is underlined by strong phenotypes of plants, deficient in factors involved in miRNA biogenesis, such as AGO1 (Bohmert et al., 1998; Morel et al., 2002), SE (Clarke et al., 1999; Grigg et al., 2005; Prigge and Wagner, 2001). Also, *hyl1hen1* double mutant plants were shown to be unable to complete a life cycle (Vazquez et al., 2004). An important role for miRNAs in plant development has been demonstrated in studies of *dcl1* mutant plants. Originally named *emb76* and *sus1* (Errampalli et al., 1991; Castle et al., 1993), null alleles of *dcl1* exhibit developmental arrest at globular stage of embryogenesis and abnormal divisions within the extraembryonic suspensor (Schwartz et al., 1994). Therefore, it was proposed that miRNAs negatively regulate expression of differentiation-promoting transcription factors which are required for proper embryonic patterning (Nodine and Bartel, 2010).

Effects of multiple plant miRNAs on developmental processes in *Arabidopsis* have been described. These effects are linked to the functions of downstream genes, transcripts of which are targeted by these miRNAs (Table 1.2). Leaf morphogenesis and patterning as well as establishment of polarity are controlled by miR319, miR165/166, miR164, miR159; developmental timing by miR156, miR172, miR390, miR159; floral organ identity is affected by miR172, miR164, miR160; and

phytohormone signaling by miR159, miR167, miR160, miR319, miR164 (Table 1.2) (Kidner and Martienssen, 2003; Wu, 2013).

miRNA	Function in plant development	miRNA targets	Developmental phenotype	Described
miR156	Floral organ identity and Developmental timing	*SPL2/3/4/5/6/ 9/10/11/13/15*	Overexpression leads to delayed vegetative development, and accelerated plastochron; oppositely, loss-of-function of miR156 miRNA causes accelerated vegetative development and early flowering	Wang, Schwab, Czech, Mica, & Weigel, 2008; G. Wu, 2006; G. Wu et al., 2009
miR159	Floral organ identity and Developmental timing Phytohormone signaling Floral organ identity	*MYB33/65/97/ 101/104/120*	Loss-of-function results in development of the hyponastic leaves phenotype. Overexpression causes hypersensitivity to ABA late flowering	Achard, Herr, Baulcombe, & Harberd, 2004; Allen et al., 2007; Reyes & Chua, 2007
miR160	Floral organ identity	*ARF10/16/17*	Loss-of-function of leads to abnormal flower development (including narrow sepals and petals, misplaced floral organs, developing from within the siliques), serrated leaves.	X. Liu et al., 2010; Mallory, Bartel, & Bartel, 2005; Wang et al., 2005
miR164	Floral organ identity and Developmental timing	*CUC1, CUC2, NAC1*	Loss-of-function causes leaf serration, development of additional petals. Overexpression of miR164 results in floral organ fusions	Laufs, Peaucelle, Morin, & Traas, 2004; Mallory, Dugas, Bartel, & Bartel, 2004; Nikovics et al., 2006; Sieber, Wellmer, Gheyselinck, Riechmann, & Meyerowitz, 2007
miR390	Developmental timing	*TAS3-non-coding RNA*	Loss-of-function causes accelerated vegetative development and affects the lateral Root Growth	Marin et al., 2010; Yoon et al., 2010

miR165/166	Leaf morphogenesis, patterning and polarity establishment	*PHB, PHV, REV, ATHB8, ATHB15*	Overexpression of miR165/166 or mutations in the miRNA binding sites in the target cause leaf polarity defects	McConnell et al., 2001; McHale & Koning, 2004; Kim et al., 2005; Williams, Grigg, Xie, Christensen, & Fletcher, 2005
miR167	Phytohormone signaling	*ARF6/8*	Overexpression of miR167 leads to development of shorter and indehiscent anther	N. Liu et al., 2014; M.-F. Wu, Tian, & Reed, 2006
miR172	Developmental timing Floral organ identity	*TOE1, TOE2, TOE3, AP2, SMZ, SNZ*	Loss-of-function of miR172a delays the vegetative-to reproductive phase change; overexpression of miR172 causes precocious vegetative development and early flowering	Jung et al., 2007; Lauter, Kampani, Carlson, Goebel, & Moose, 2005; Milo J Aukerman, 2003; G. Wu et al., 2009
miR319	Leaf morphogenesis, patterning and polarity establishment; Phytohormone signaling	*TCP2/3/4/10/24*	Overexpression of this miRNA results in aberrant curling and serration of the leaves, also it reduces the jasmonic acid level and delays leaf senescence	Palatnik et al., 2003; Schommer et al., 2008

Table 1.2 Roles of miRNAs in plant development

The table represents a list of plant miRNAs and miRNA targets, which were reported to affect plant development. It includes the description of developmental phenotypes of plants, deprived of these miRNAs or overexpressing them (Wu, 2013).

Transcriptional regulation on miRNAs expression

The first miRNA was identified in *C. elegans* in a forward genetic screen designed to study genes that cause defects in timing of larval development (Lee et al., 1993; Wightman et al., 1993).

Unlike animal miRNAs, often derived from introns or untranslated regions (Carthew and Sontheimer, 2009), plant miRNA coding genes are only rarely located within protein-coding genes. The precursors of miRNAs are transcribed by RNA polymerase II (Pol II) (Lee et al., 2004).

The abundance of a mature miRNA depends on level of transcription of its

MIRNA gene and the activity by which it is processed. Accumulation of miRNAs was shown to be affected in mutants of components of miRNA biogenesis (Kurihara, 2005; Fagard et al., 2000). At the same time, less is know about factors directly controlling transcription of miRNA genes.

Recently, it was reported that the cell division cycle 5 (CDC5) protein, a MYB transcription factor related protein conserved in animals, plants, and fungi (Ohi et al., 1998), functions as a positive regulator of transcription and processing of a number of precursor miRNAs (pri-miRNAs) (Zhang et al., 2013). It directly interacts with RNA Pol II and the promoters of miRNA genes, influencing the transcription of target loci. In addition CDC5 also associates with DCL1 participating in the regulation of miRNA maturation (Zhang et al., 2013). Recruiment of RNA Pol II to promoters of miRNA genes was also shown to be facilitated by the Mediator complex and the coactivating factor NOT2b (Kim et al., 2011). Similar to CDC5, NOT2b has been reported to mediate recruitment of DCL1 to promote miRNA biogenesis (Wang et al., 2013).

On the other hand, transcription of certain miRNA family members has been shown to be regulated by the transcription factors POWERDRESS (PWD) and FUSCA 3 (FUS3). PWD was reported to affect transcription of *MIR172A*, and FUS3 has been detected in the promoters of *MIR156A* and *MIR156C* (Wang and Perry, 2013; Yumul et al., 2013).

Trans-acting siRNAs

Similar to miRNAs, trans-acting small RNAs (ta-siRNAs), regulate gene expression. Precursor transcripts of ta-siRNAs, encoded by *TAS* genes, do not code for proteins, however they are capped and polyadenylated (Vazquez and Hohn, 2013). *TAS* RNAs contain miRNA recognition sites. Targeting of a *TAS* transcript by the corresponding miRNA triggers its cleavage and further processing into ta-siRNAs (Howell et al., 2007). Thus, the production of ta-siRNAs also depends on components of the miRNA biogenesis pathway, such as DCL1 and AGO1. The cleavage products are stabilized by SUPPRESSOR OF GENE SILENCING 3 (SGS3) (Mourrain et al., 2000). Then the RNA DEPENDENT RNA POLYMERASE 6 (RDR6) is recruited to produce a second RNA strand of the precursor transcript (Ronemus et al., 2006;

Harmoko et al., 2013; Wassenegger and Krczal, 2006). AGO7 has been shown to participate at this stage (Hunter et al., 2003; Hutvagner and Simard, 2008; Montgomery et al., 2008) (Figure 1.5).

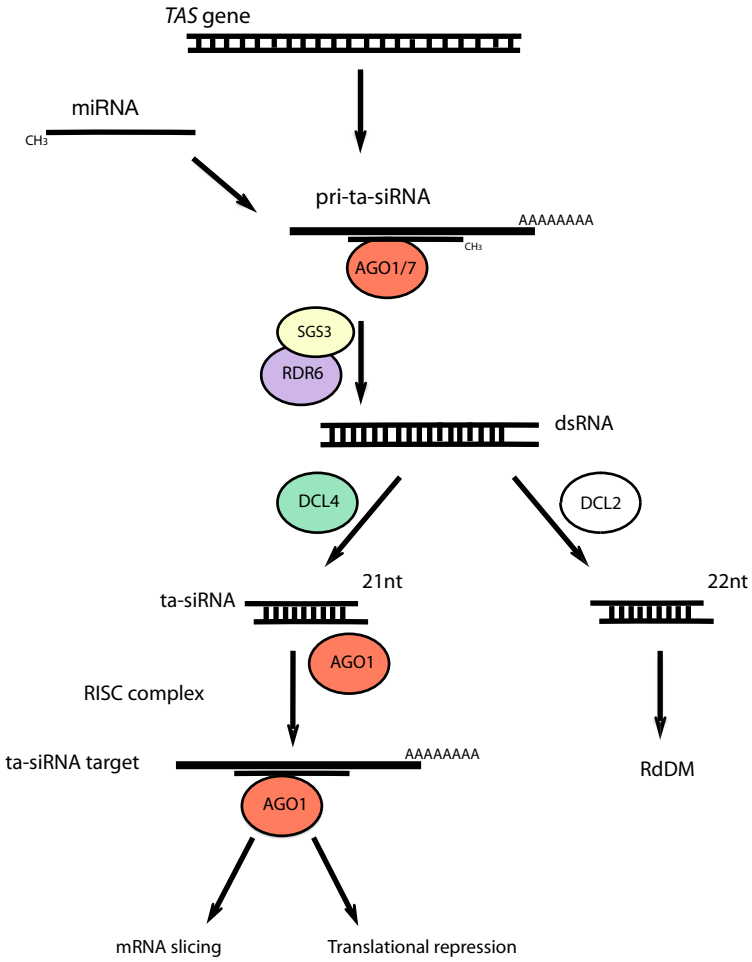


Figure 1.5 Pathway of ta-siRNA production

The precursor ta-siRNA transcripts derive from dedicated *TAS* genes. These transcripts contain binding sites for miRNAs leading to cleavage of the *TAS* transcript. These cleavage products are recognized and bound by SGS3, AGO7 and RDR6 to stabilize ta-siRNA precursors and to synthesize the complementary RNA strand. The resulting dsRNA in then recognized and cleaved by DCL4 to 21-nt ta-siRNA, which are loaded in AGO1 and further trigger the cleavage of complementary mRNA. Cleavage of the dsRNA by DCL2 leads to production of 22-nt sRNAs, which further mediate the establishment of DNA methylation via RdDM pathway.

The resulting double-stranded RNA (dsRNA) is further cleaved into 21-nt long ds-RNA duplexes by DCL4 and its interacting partner dsRNA BINDING protein 4 (DRB4) (Allen et al., 2005; Nakazawa et al., 2007; Xie et al., 2005) or, alternatively into 22-nt fragments by DCL2 (Gasciolli et al., 2005; Mlotshwa et al., 2008) (Figure 1.6). Further, similarly to miRNAs, the 21-nt ta-siRNAs are methylated by HEN1 (Gasciolli et al., 2005). Then, mature ta-siRNAs are incorporated in RISC with AGO1 to further mediate cleavage of complementary mRNA (Figure 1.5) (Baumberger and Baulcombe, 2005; Mallory and Vaucheret, 2010). DCL2-dependent 22-nt sRNAs are further involved in RdDM pathway (described previously) and play an important role in transitive silencing of transgenes (Mlotshwa et al., 2008).

Important role of ta-siRNAs in plant development can be demonstrated on the example of ta-siRNAs derived from *TAS3* gene, which is targeted by miR390. Mature *TAS3* ta-siRNAs further target mRNAs of the AUXIN RESPONSE FACTOR (ARF) transcription factor family for degradation (Allen et al., 2005; Williams et al., 2005; Fahlgren et al., 2006). ARFs regulate expression of auxin-responsive genes by binding to their promoters (Ulmasov, 1997; Ulmasov et al., 1999). It was shown that transgenic plants, expressing a ta-siRNA insensitive version of *ARF3* exhibit strong leaf phenotype and accelerated phase change. Therefore, regulation of *ARF3* by *TAS3* ta-siRNAs affects developmental timing and patterning in *Arabidopsis* (Fahlgren et al., 2006).

Recent studies of the differences in activities of miRNA and ta-siRNA suggest that functional distribution between these regulatory components might be connected to their properties. The mobility of miRNAs is limited to short distance within neighboring cells, whereas ta-siRNAs may have higher movement capacities, allowing them to act in distant tissues (Chitwood et al., 2009).

1.5 miR156 is an important regulator of plant development

miR156 and developmental phase change

Three main phases – juvenile vegetative, adult vegetative and reproductive – can be distinguished during plant development (Poethig, 1990). The separation between these phases can be drawn based on specific morphological characteristics. Leaf shape and size, leaf arrangement, patterns of epidermal differentiation and trichome distribution characterize the age of a plant during juvenile and adult vegetative phases (Poethig, 2003). In *Arabidopsis* the change between juvenile and adult phases is marked by a change in distribution of trichomes on the surfaces of leaf plates. The trichomes are present only on adaxial (upper) surface of the early rosette leaves (juvenile), whereas adult rosette leaves have trichomes on both surfaces (Telfer et al., 1997). The further transition from vegetative to reproductive phase requires the reprogramming of the shoot apical meristem (SAM) and its transformation into inflorescence meristem (IM) with the capacity to produce reproductive organs (Willmann and Poethig, 2005; Wiltshire et al., 1994). For proper timing and coordination of the developmental phase transitions, the integration of information obtained from environmental and endogenous sources is required (Poethig, 1990; Amasino, 2010; Jung et al., 2011). In *Arabidopsis,* miR156 has been shown to be one of the regulators of developmental phase transitions (Wu, 2006).

Highly conserved throughout plant kingdom (Axtell and Bowman, 2008), miR156 is one of the most abundant miRNAs in *Arabidopsis* (Wang et al., 2009; Kozomara and Griffiths-Jones, 2011). In the genome of *Arabidopsis*, at least 10 *MIR156* family members (including *MIR156I* and *MIR156J*) have been identified (Kozomara and Griffiths-Jones, 2011; Breakfield et al., 2012).

Basal expression level has been shown to differ a lot within *MIR156* family genes. By Yang et al., 2013, the expression of *MIR156F* and *MIR156H* was reported to be significantly lower than that of *MIR156A – D* and transcripts of *MIR156E* and *MIR156G* were not detected by qPCR. Both isoforms (*A* and *C*) have the time-dependent trend of decrease in expression (correlating with the dynamics of abundance of 20-nt miRNA) (Yang et al., 2013).

Recent studies suggest that different miR156 isoforms display tissue specific patterns of expression. High resolution profiling of *Arabidopsis* miRNAs in roots

revealed that *MIR156A* and *MIR156H* are expressed in cortex and epidermis tissues in late meristematic and elongation zones of a root (Breakfield et al., 2012). On the other side, GUS reporter lines revealed strong expression driven by the promoters of *MIR156A* and *MIR156C* in the vasculature and hypocotyl of young seedlings (Yang et al., 2013). In reproductive organs, expression of *MIR156C -D* and *-H* overlapped with expression of their target genes in anther development (Xing et al., 2010). Expression of *MIR156H* was also observed in ovules (Xing et al., 2011).

miR156 has a time-dependent expression profile reaching its maximum at the seedling stage, followed by a gradual decrease during ageing of the plant (Wu et al., 2009, Figure 1.6 A). It is mainly represented by siRNAs of 20 and 21 nucleotide forms. Interestingly, abundance of the 20 nt form is time-dependent and decreases with development of a plant, whereas level of the 21-nt form stays constant (Wu, 2006; Wu et al., 2009). Constitutive overexpression of *MIR156* (*35S::miR156*) causes production of larger number of rosette leaves and moderate delay in flowering time (Wu, 2006). The plants with decreased miR156 level have an accelerated juvenile-to adult phase transition and early flowering (Franco-Zorrilla et al., 2007; Todesco et al., 2010).

However, the mechanism, by which such gradual decline is regulated, remains unclear (Yang et al., 2013). Recent reports suggest that a signal, negatively regulating the level of miR156 might be produced by leaves (Yang et al., 2010). Abundance of miR156 was also reported to be influenced by temperature, CO_2 (May et al., 2013) and sugar (Yang et al., 2013).

Expression of genes, transcripts of which are targeted by miR156 also has a gradual trend opposite to the one of the microRNA, increasing with the age of a plant (Wu, 2006; Wu et al., 2009; Wang et al., 2009; Yamaguchi and Abe, 2012) (Figure 1.6 A). It is supposed that the major mode of action of miR156 is mRNA degradation and in some cases translational inhibition (Brodersen et al., 2008; Gandikota et al., 2007). However, results of recent studies implicate that, in addition to 20- and 21 nt forms, *MIR156* genes can give rise to 23- to 26-nt siRNAs (Chellappan et al., 2010). While 20 – 21-nt miRNAs, mainly associate with AGO1 thereby targeting transcripts for degradation or suppression of protein synthesis, 23- to 26-nt siRNAs derived from a *MIR156* locus can associate with AGO4 and mediate establishment of DNA methylation at their target sites (Chellappan et al., 2010). Therefore, siRNAs derived

from *MIR156* can influence expression of target genes at both transcriptional and post-transcriptional levels.

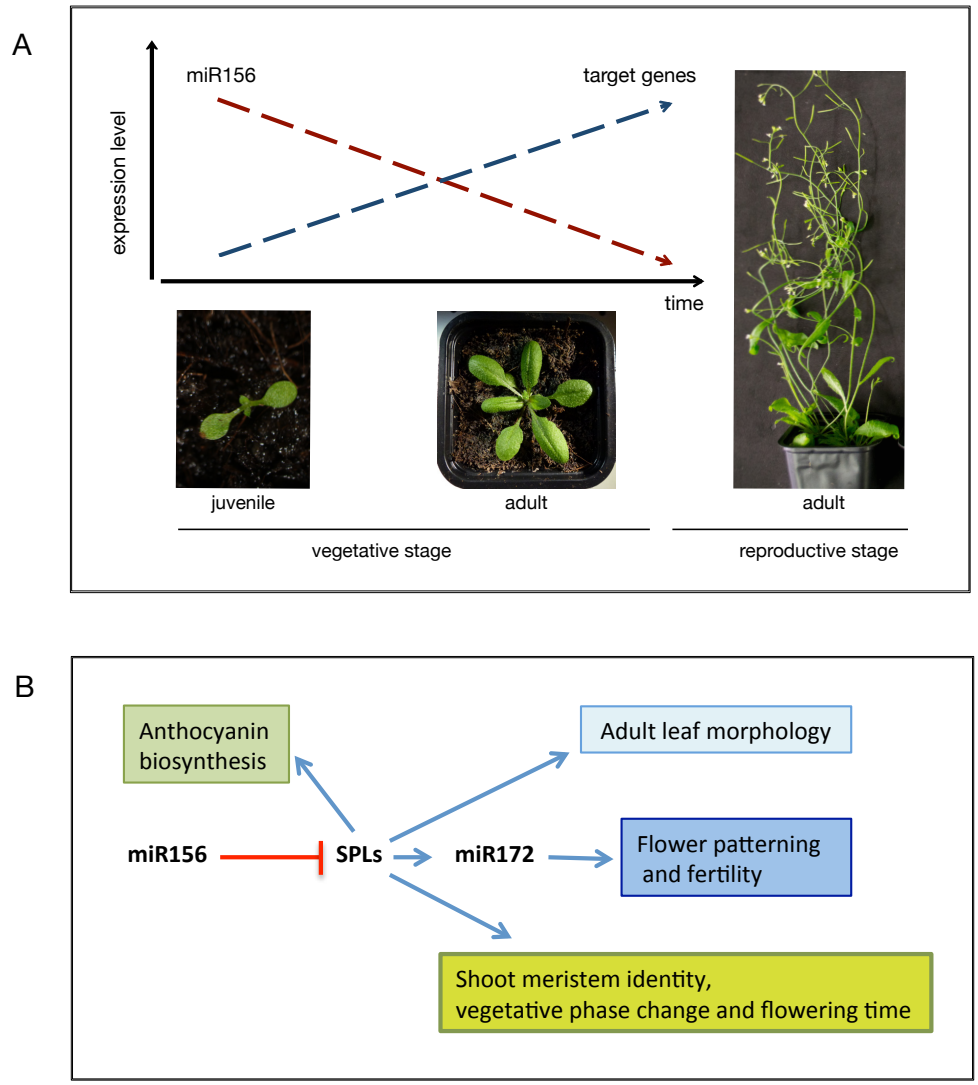


Figure 1.6 Role of miR156 in developmental phase change

(A) Expression of *MIR156* has a time-dependent profile with its maximum at a seedling stage, gradually declining with the age of a plant. The expression of the genes targeted by miR156 has a gradual increasing trend that is opposite to that of the microRNA. miR156 has been shown to regulate the timing of developmental phase transitions in *Arabidopsis*. (B) The group of *SPL* transcription factors that is targeted by miR156 includes important regulators of developmental phase change, anthocyanin biosynthesis, leaf morphology, flower patterning and fertility, and shoot apical meristem identity linking miR156 to these pathways.

Targets of miR156 are important developmental regulators

Targets of miR156 have been identified as important regulators of developmental phase change. Transcripts of several plant-specific *SQUAMOSA PROMOTER BINDING PROTEIN LIKE* (*SPL*) transcription factors are targeted by miR156 (Cardon et al., 1999; Chen et al., 2010; Guo et al., 2007; Yamaguchi and Abe, 2012; Xing et al., 2011). SPLs bind DNA in a sequence specific manner by recognizing a GTAC core sequence in promoter regions of the target genes (Cardon et al., 1999; Birkenbihl et al., 2005). They contribute to control of major developmental processes, such as seed germination, juvenile-to-adult and floral phase transitions (Gandikota et al., 2007; Nonogaki, 2010; Shikata et al., 2009), leaf and plastocron development (Wang et al., 2009), trichome formation (Yu et al., 2010), and fruit ripening (Manning et al., 2006) (Figure 1.6 B).

In *Arabidopsis, SPL* genes are divided into 2 main groups (groups I/II Figure 1.8) based on genomic organization and transcript size (Yamasaki et al., 2004; Xing et al., 2010). 5 genes, belonging to the first group (*SPL1, SPL7, SPL12, SPL14, SPL16*) encode large proteins (over 750kDa), whereas the second group includes smaller and less complex proteins of up to 400kDa. Except for *SPL8*, all members of the second group (*SPL2, SPL3, SPL4, SPL5, SPL6, SPL9, SPL10, SPL11, SPL13a, SPL13b* and *SPL15*) contain a miRNA156 recognition site (Reinhart, 2002; Schwab et al., 2005; Guo et al., 2007). *SPL* genes can be further divided to subgroups, based on sequence similarities (Xing et al., 2010; Yamasaki et al., 2004) (Figure 1.7).

Recent studies revealed, that miR156 targeted *SPL* genes are involved in the regulation of leaf development and vegetative phase change (Wu, 2006; Wu et al., 2009). SPL3 directly regulates expression of the flower meristem identity genes *LEAFY (LFY), APETALA1 (AP1), FRUITFULL (FUL)* and *FLOWERING LOCUS T (FT)* (Yamaguchi and Abe, 2012; Kim et al., 2012). Whereas SPL9 has been reported to be a positive regulator of *MIR172B* expression, linking the action of miR156 and miR172 (Wu et al., 2009). Constitutive overexpression of the miR156-resistant forms of *SPL3, SPL4 or SPL5* leads to acceleration of juveline-to-adult phase transition and early flowering (Wu, 2006; Kim et al., 2012). At the same time, plants overexpressing the miR156-resistant forms of *SPL9* or *SPL15* demonstrate changes in cell number

and cell size, a short juvenile phase and early flowering (Usami et al., 2009; Schwarz et al., 2008).

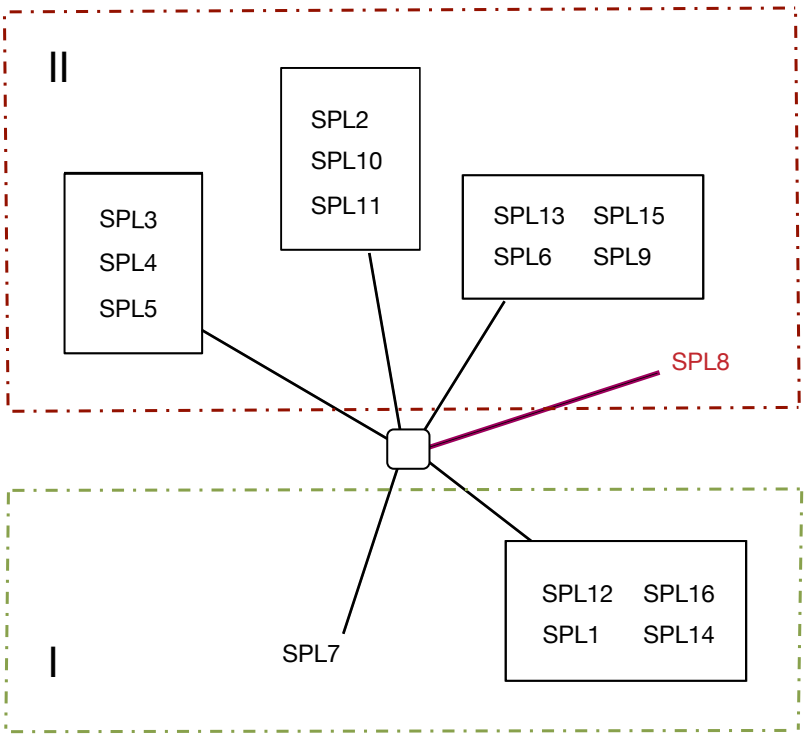


Figure 1.8 Phylogenetic relationships among the *SPL* genes of *Arabidopsis*

Scheme is based on unrooted phylogram of all *SPL* genes published by (Xing et al., 2011). Two large families of *SPL* genes (I and II) are marked with dashed lines. Members of family I are mainly large genes, not containing the miR156 target site, whereas genes belonging to the family II, are targeted by this microRNA. An exception is *SPL8,* which is structurally related to family II but not containing the miR156 target site.

SPL8, which structurally belongs to group II of the *SPL* transcription factors, does not contain a miR156 target site and is required for pollen sac development and trichome initiation on sepals (Zhang et al., 2006). Plants defective for SPL8 display severe sterile flower phenotypes (as a result of abnormalities in pollen sac formation) at the early flowering stage (first flowers). However, fertility recovers in later flowers,

thus indicating partial compensation of loss of function of SPL8 by other SPL genes (Xing et al., 2010). Therefore it was suggested that synchronized action of multiple miR156-targeted *SPL* genes and *SPL8* are required for development of fully fertile flowers (Xing et al., 2013). In support to this assumption, early flowers of plants with ectopic expression of *SPL3* did not display developmental abnormalities while the later flowers were strongly affected (Gandikota et al., 2007).

Recent studies show a possibility of feedback regulation of miR156 precursor transcription by SPLs (Fornara and Coupland, 2009; Wei et al., 2012). Such feedback connection may be part of a mechanism which determines the irreversibility of developmental phase transitions (Wang et al., 2009; Yu et al., 2012). However, while the events downstream of *MIR156* transcription have been described, not much is known about the mechanisms that directly control the transcription of *MIR156* (Huijser and Schmid, 2011; Yamaguchi and Abe, 2012).

1.6 The Aims of this Thesis

Multiple mechanisms are acting in concert to assure dynamic regulation of gene expression. Among them are: DNA methylation, histone modification and chromatin remodeling. Recent reports indicated multiple connections between pathways of transcriptional and post-transcriptional gene silencing. At the same time, impact of individual mechanisms on regulation of gene expression at a tissue specific level remains to be studied.

Previously, it was reported that Pol V and MORPHEUS' MOLECULE1 (MOM1) have a synergistic effect of on silencing of the *APUM9* (At1g35730) (Yokthongwattana et al., 2010). It was suggested that expression of *APUM9* may be affected by transcriptional silencing of the ROMANIAT5 TE, located in the proximity of the gene (Yokthongwattana et al., 2010). However, the precise mechanisms, defining such regulation remained to be investigated.

Therefore, a study was designed by E. Bucher to identify new epigenetic factors, which contribute to regulation of the *APUM9* expression in *Arabidopsis*. In order to better investigate the mechanisms, involved in regulation of *APUM9* expression, a transgenic GFP reporter line, containing the endogenous *APUM9* promoter was chosen.

The aim of this thesis project was to investigate the mechanisms, involved in tissue specific silencing of the GFP transgene in the chosen transgenic line. To identify novel epigenetic factors contributing to this process a forward genetic mutant screen was set up. Following general characterization of the transgenic line, the project was mainly focused on characterization of one mutant, recovered in the mutant screen.

CHAPTER II Characterisation of the APUM9 reporter line

INTRODUCTION

Successful survival and propagation of an organism often requires drastic changes in morphological and physiological characteristics throughout development. It is particularly important for plants as they are more exposed to changes in environmental conditions and that they are sessile. At the molecular level, signals from external and internal factors trigger changes in expression of responsible genes, which result in generation of further responses to that stimulus.

Regulation of gene expression through silencing can take place at transcriptional (before initiation of transcription) or at post-transcriptional (after the mRNA transcript has been formed) level. Mechanisms of transcriptional gene silencing (TGS) include establishment of DNA methylation, covalent modifications of histones and also changes in the chromatin structure by remodeling complexes. Post-transcriptional gene silencing (PTGS) mechanisms act by cleavage of mRNA transcripts or translational repression.

Existing physiological and functional differences between tissues imply that different sets of genes need to be expressed or repressed. Consequently, that would require the presence of mechanisms regulating gene expression in a tissue-dependent manner. While the studies of such mechanisms are being actively performed in animals (Kizuka et al., 2014; Przybilla et al., 2014; Rosa et al., 2014), research of this area in plants is currently less advanced. Recent reports proposed existence of a tissue-specific epigenetic code in plants (Caro et al., 2007).

Earlier, in studies about the impact of Pol V (a component of RdDM) and MOM1 (acts in TGS without affecting the DNA methylation) on transcriptional gene silencing, the *APUM9* (At1g35730) gene was identified to lie under synergistic control by both factors. The expression of *APUM9* was shown to be activated in *mom1* mutants and further enhanced in *mom1nrpe1* double mutant plants (Yokthongwattana et al., 2010). Also, it was demonstrated that transcription of *APUM9* may be driven by the Long terminal repeat (LTR) of a ROMANIAT5 retrotransposon located in its promoter region. Thus, transcriptional silencing of the ROMANIAT5 may affect expression of *APUM9* (Yokthongwattana et al., 2010,

Zemach et al., 2013). However, the mechanisms defining such regulation remained to be investigated.

APUM9 belongs to a family of PUF of RNA-binding proteins, containing the Pumilio homology domain (PUM-HD, Tam et al., 2010). The PUF proteins, identified in plants exhibit 50-70% of sequence similarities with the *Drosophila PUMILIO* (*PUM*) gene, which encodes a protein that play roles in germline development, gonadogenesis, oogenesis and embryogenesis (Barker et al., 1992; Francischini and Quaggio, 2009; Parisi and Lin, 1999). Until recently, only limited information on their phylogenetic connections and molecular functions was available (Spassov and Jurecic, 2003; Wickens et al., 2002).

It has been reported that putative targets of translational regulation by homologues of *APUM9* include WUSCHEL (WUS) and FASCIATA-2 (FAS-2) (Francischini and Quaggio, 2009), Also, *APUM23*, another member of this family, has been shown to function in leaf development and organ polarity in *Arabidopsis* (Huang et al., 2014). This indicates possible functions of *APUM9* in regulation of growth and development.

Therefore, to further study the epigenetic mechanisms contributing to regulation of *APUM9* expression, a transgenic GFP reporter line was used. The chosen transgenic line, was received from a collection of GFP reporter lines (line AGRAC-60-1-1) (Xiao et al., 2010). The reporter construct contains the 2.3kb of the endogenous *APUM9* promoter including 75 bp of *APUM9* CDS and 1.5 kb of the *ROMANIAT5* retrotransposon, located upstream of the *APUM9* promoter (Figure 2.1 A). The transgene is based on a two component reporter system: The *APUM9* promoter drives transcription of the *GAL4-VP16* transcriptional activator (Sadowski et al., 1988). GAL4-VP16 then binds to the *UAS* (Upstream Activation Sequence) to drive GFP expression (Haseloff, J., 1999) (Figure 2.1 B). Similar reporter systems have previously been successfully applied in studies of gene functions in several model organisms: *Arabidopsis* (Engineer et al., 2005), rice (*Oryza sativa L.*) (Liang et al., 2006) *Drosophila* (Fischer et al., 1988; Brand and Perrimon, 1993), zebrafish *(Danio rerio)* (Scheer and Campos-Ortega, 1999; Scheer et al., 2002), and Japanese rice fish (*Oryzias latipes*) (Grabher and Wittbrodt, 2004).

Previous results of classic mapping, segregation analysis and whole genome sequencing confirmed that the transgene of the silex line is the result of a single transgene insertion located on the upper arm of chromosome 3 (within the second

intron of AT3G07640 encoding for an unknown protein). Plants of this transgenic line exhibited no GFP expression in seedlings or vegetative organs of adult plants, however later GFP expression was observed in the valve margin of siliques (Figure 2.1). Following this observation, the line was named "silex" (siliques expression).

Therefore, the general goal of this chapter was to investigate the contribution of different gene-regulatory mechanisms to tissue specific gene silencing of GFP expression in the silex line.

RESULTS

The transgenic silex line displayed highly tissue-specific GFP expression pattern observed (Figure 2.1 C). It has previously been reported that transcription of the endogenous *APUM9* gene is controlled by at least two independent TGS pathways (Yokthongwattana et al., 2010, Zemach et al., 2013). Also, it was suggested that transcriptional silencing of the *ROMANIAT5* may affect the expression of the endogenous *APUM9* (Yokthongwattana et al., 2010; Zemach et al., 2013). As loss of DNA methylation has been reported to coincide with the activation of *APUM9* expression (Yokthongwattana et al., 2010; Zemach et al., 2013), we hypothesised that it might also play a role in silencing the GFP transgene in the silex. Therefore, to investigate the mechanisms involved in silencing the GFP transgene in silex, we first checked the effect of removal of DNA methylation on GFP expression. For this the silex line was grown on MS medium supplemented with 40 mM Zebularine, a drug that inhibits DNA methylation (Zhou et al., 2002; Cheng et al., 2003; Marquez et al., 2005; Baubec et al., 2009). GFP expression was released in cotyledons of plants, treated with Zebularine in a stochastic manner; whereas no GFP expression was detectable in cotyledons of untreated plants of the same age (Figure 2.2 A). This indicates that DNA methylation indeed plays a role in silencing GFP expression in silex.

To further study the role of TGS in repression of the GFP transgene, silex was crossed with *nrpe1* (deficient in Pol V activity, required for targeting of DNA methylation in the CHH context) (Haag and Pikaard, 2011; Kanno et al., 2005; Onodera, 2005). Plants homozygous for *nrpe1* and containing the GFP transgene, did not display visible release of GFP expression in seedlings and young plants. To check for changes at the CHH methylation level in the promoter region of the transgene, genomic DNA was extracted from 18days old plants of silex and *nrpe1* and digested with the methylation sensitive restriction enzyme *DdeI*. This enzyme cuts DNA at the –C'TNAG– recognition sequence and therefore reports on DNA methylation in CHH context. Presence of DNA methylation at the *DdeI* restriction sites prevents digestion of DNA, allowing it to be amplified by PCR. Consequently, absence of methylation at the *DdeI* restriction sites results in that no PCR amplification of the region. Digested and non-digested DNA (as a control) was then PCR-amplified with primers, specific

for the promoter of transgene (location of primers is indicated in Figure 2.1B, sequences of primes are listed in Appendix I). The absence of a PCR product in *nrpe1* indicates loss of CHH methylation at the transgene (making it available for restriction) (Figure 2.2 B-C).

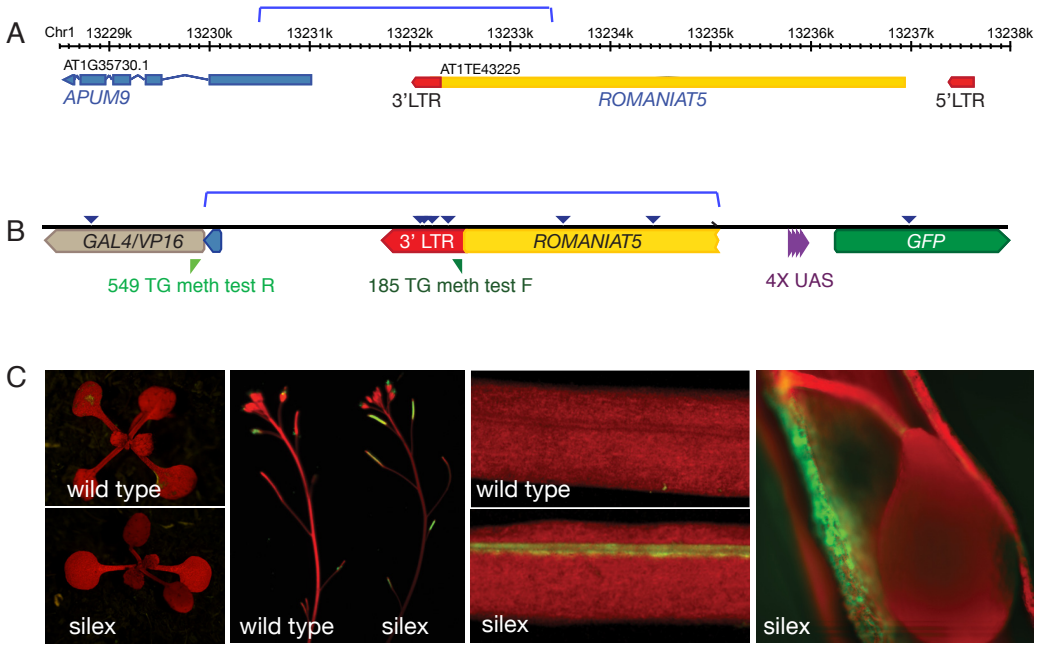


Figure 2.1 Structure of the transgene in silex line

(A) Schematic representation of the endogenous *APUM9* locus on chromosome 1 of *Arabidopsis*. The exons of *APUM9* are indicated by blue boxes. The yellow box upstream of *APUM9* marks the *ROMANIAT5* retrotransposon and the red boxes represent its LTRs. The green line on the top indicates the promoter sequence used for the transgene in the silex line. (B) Schematic representation of the GFP transgene, containing 2395 bp of the DNA sequence upstream of the CDS including 75 bases of the *APUM9* CDS. It was cloned in front of *GAL4/VP16*, which in turn will recognise the 4X *UAS* sequence in front of *GFP* to drive GFP expression. The blue triangles above the DNA sequence indicate *DdeI* restriction sites. (C) Fluorescence images of the silex reporter line. GFP expression is green and chloroplast autofluorescence is red. Left panel shows 18 days old plants of wild type (Col) and silex with no detectable GFP expression. Central panels show GFP expression in siliques (with the zoomed image of valve margin of siliques where the GFP was detected). A dissected silique is shown on the right panel depicting a seed and the green fluorescent valve margin. Wild type non-transgenic plants are shown as controls.

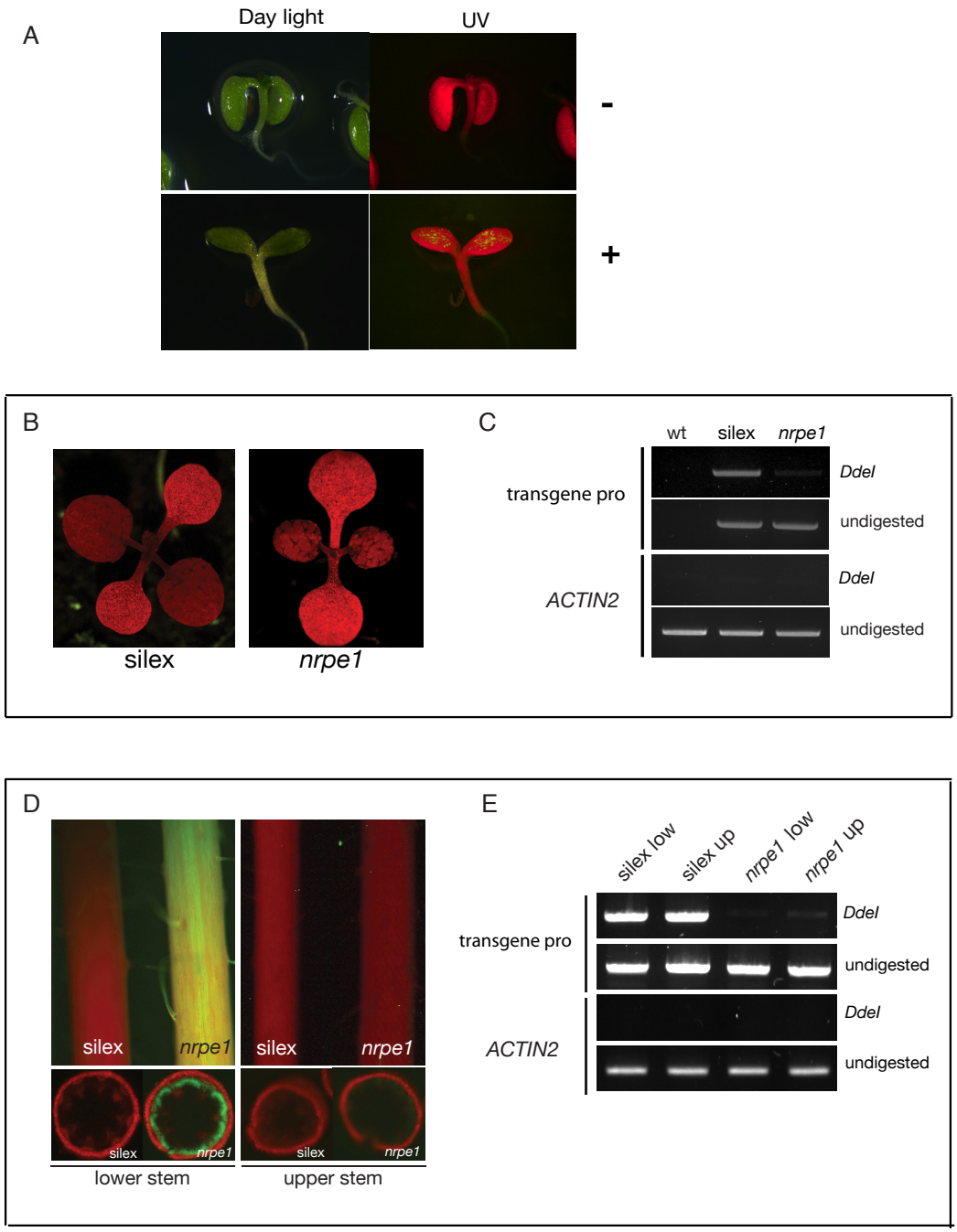


Figure 2.2 Loss of methylation influences GFP expression in silex

(A) Treatment of the silex line with 40 μM Zebularine releases GFP silencing. Daylight and fluorescence images of cotyledons of non-treated silex plants (-) and plants treated with Zebularine (+). (B) Fluorescence images of *Arabidopsis* seedlings, showing that no GFP expression is visible in seedlings of *nrpe1,* containing the silex transgene. (C) PCR based DNA methylation assay on DNA from *Arabidopsis* seedlings, showing the reduction of DNA methylation in promoter of silex transgene due to loss of NRPE1 function. Genomic DNA extracted from 18 days seedlings was digested with the methylation sensitive *Dde*I restriction enzyme followed by PCR amplification of either the silex promoter. Undigested genomic DNA was also

amplified as a positive control. To test for completed digestion, *ACT2* was amplified from digested DNA, as *ACT2* contains *Dde*I restriction sites that are not methylated. Primers, used for PCR amplification, are listed in Appendix I. (D) Fluorescence images of *Arabidopsis* stems showing release of silex transgene silencing in lower (left panels) and upper (right panels) parts of stems in *nrpe1*. The top panels show a view from the side and the two panels below represent cuts of stems viewed from the top. (E) PCR based DNA methylation assay on DNA from *Arabidopsis* stems, showing that loss of NRPE1 function reduced DNA methylation levels in the silex transgene promoter. Genomic DNA extracted from lower and upper parts of stems and further processed, as described for Figure 2.2 C.

Noticeably, release of GFP expression was observed in the basal parts of stems of adult *nrpe1* plants. To test whether release of silencing in stems correlated with loss of DNA methylation at CHH sites within the transgene promoter we performed methylation sensitive PCR. Genomic DNA was extracted from lower (from the base 3 cm up) and upper part (3 cm down from inflorescence) of stems and digested with *DdeI*, as described for seedlings. Results of PCR amplification of the transgene promoter region show that, similarly to seedlings, *nrpe1* displayed loss of CHH methylation in all tested tissues (Figure 2.2 D-E).

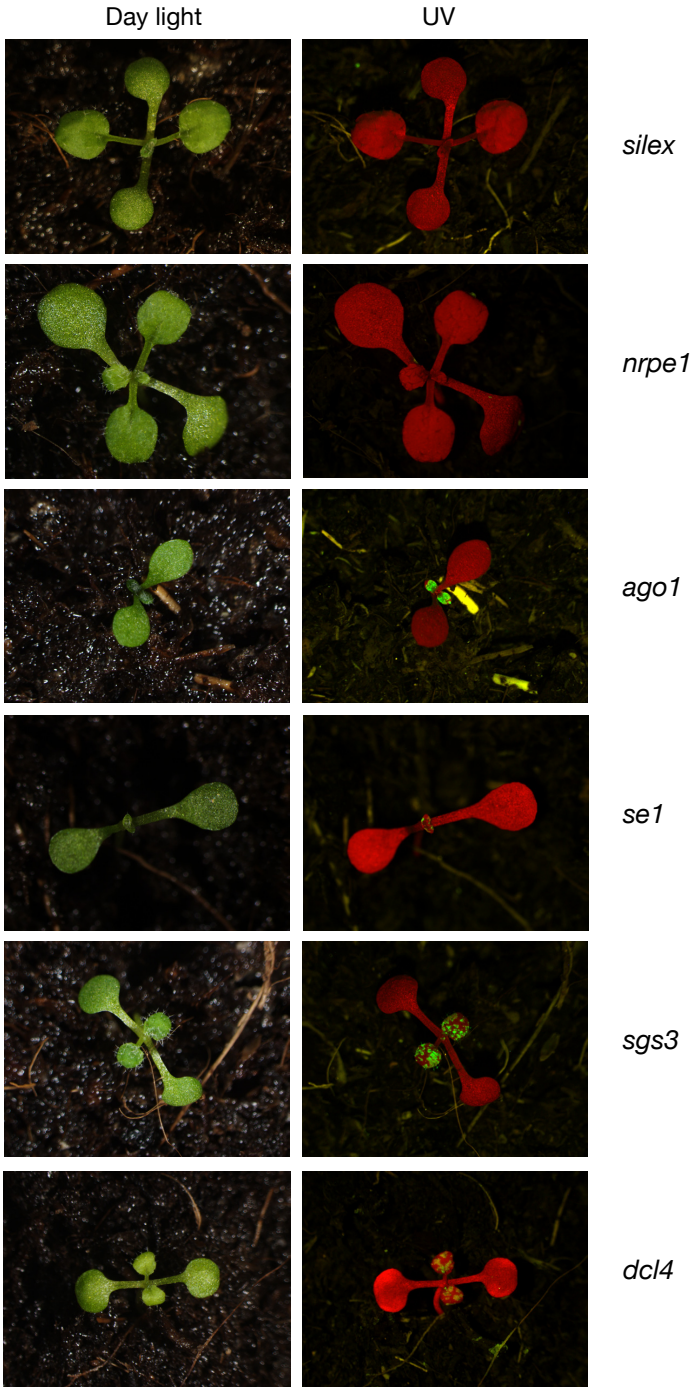


Figure 2.3 Components of the RNA silencing pathways are required to silence GFP in the silex reporter line

Daylight and fluorescence images of 12 days old plants of silex and homozygous mutants of components of silencing pathways. GFP expression is released in young leaves of *ago1*, *se*, *sgs3* and *dcl4* mutant plants.

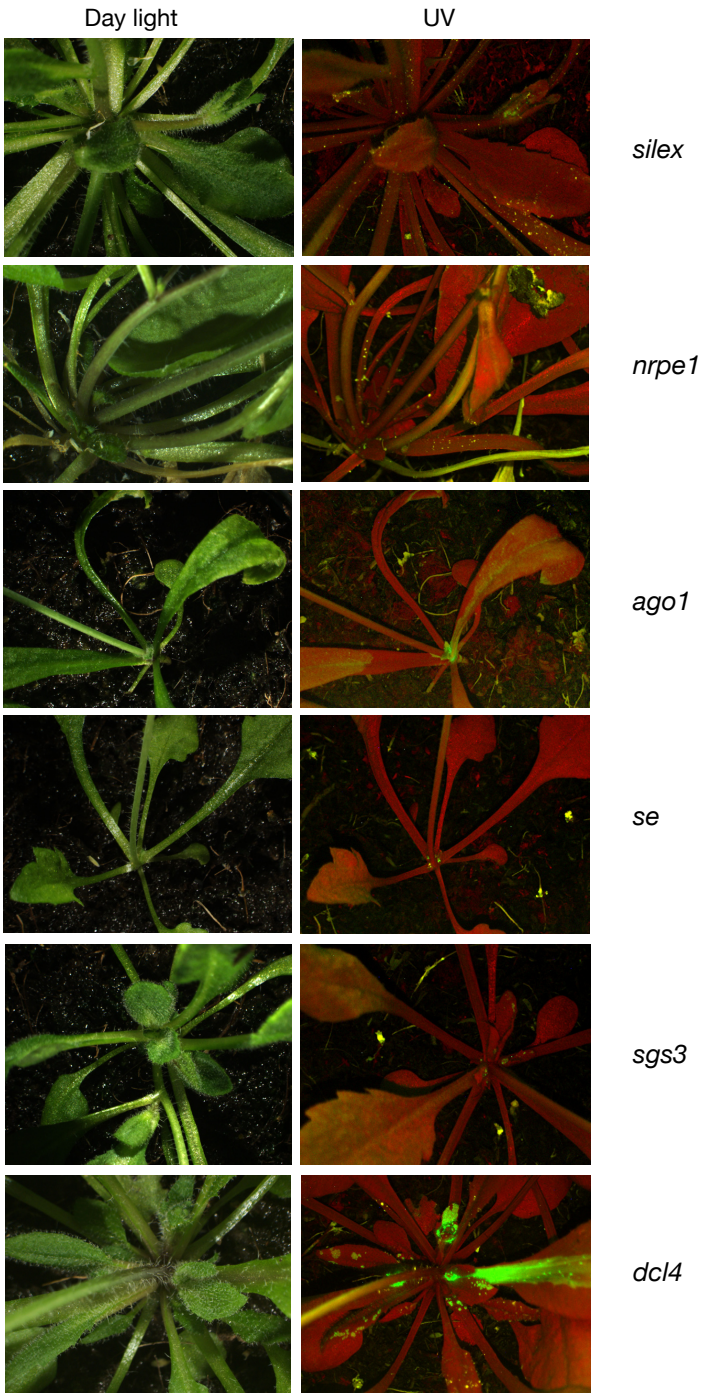


Figure 2.4 Components of silencing pathways affect GFP expression in adult leaves

Daylight and fluorescence images of rosettes from adult plants of silex and homozygous mutants of components of silencing pathways. GFP expression is observed in leaf plates of *ago1*, *se*, *sgs3* and *dcl4* homozygous mutant plants, containing the silex transgene. Release of GFP expression in inflorescence stems is exhibited by *nrpe1* (as shown previously), *ago1*, and *dcl4* mutant plants, containing the silex transgene.

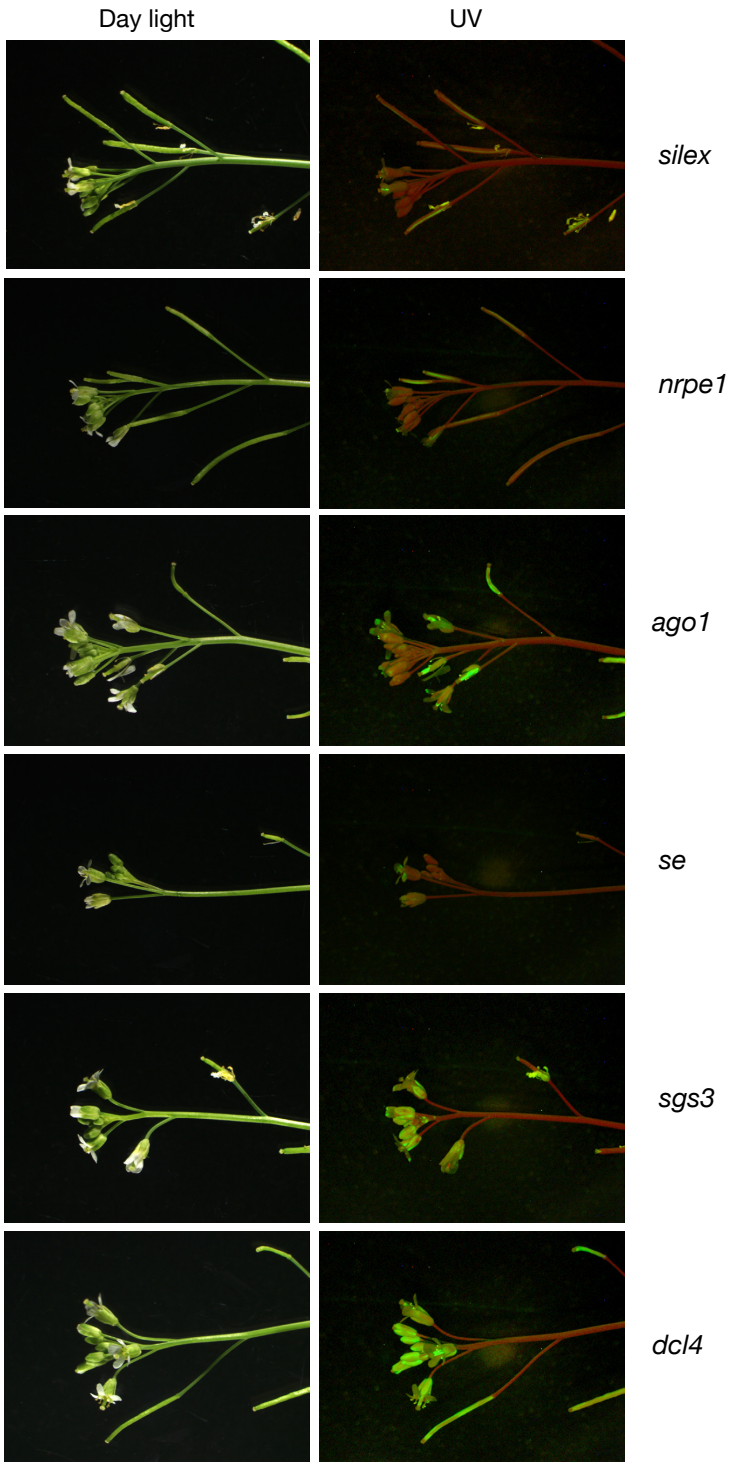


Figure 2.5 Components of silencing pathways affect GFP expression in flowers

Daylight and fluorescence images of primary inflorescences of silex and homozygous mutants of components of silencing pathways: *ago1*, *se*, *sgs3* and *dcl4*, showing release GFP expression in inflorescences *ago1*, *sgs3* and *dcl4*.

To test, whether PTGS is also involved in silencing the GFP transgene, we crossed silex with *ago1* (required for miRNA biogenesis, ta-siRNA production and DNA methylation at certain sites (Hutvagner and Simard, 2008; Yoshikawa, 2013; Wu et al., 2012). Silex plants, homozygous for mutation in *AGO1* exhibited strong release of GFP expression in young leaves (Figure 4). This suggested that PTGS may also play a role in silencing the transgene in silex. To better understand the underlying mechanisms, which regulate the GFP transgene silencing, we crossed silex with mutants of components of miRNA biogenesis (*se*) and ta-siRNA production (*sgs3* and *dcl4*).

Weak release of GFP expression was observed in young leaves of *se* plants (Figure 2.3). Which may indicate, that the microRNA pathway is involved in silencing the GFP transgene in silex.

At the same time, silex plants, homozygous by mutations in *SGS3* and *DCL4*, components of ta-siRNA pathway, also had a strong release of GFP expression in young leaves (Figure 2.3).

Adult plants of silex, carrying mutations in *SE, AGO1, SGS3,* or *DCL4,* exhibited release of GFP expression in adult leaves (although, weak for *se*). Strong GFP expression was also detected in basal parts of stems of *ago1* and *dcl4* plants (comparable to *nrpe1*, mentioned previously) (Figure 2.4). Noticeably, release of GFP expression was also observed in inflorescences of *ago1*, *sgs3* and *dcl4* (Figure 2.5) but not in *nrpe1* and *se* (Figure 2.5).

Our results suggest that components of both the TGS and the PTGS pathways are required to silence GFP expression in different tissues.

DISCUSSION

As previously reported, the expression of endogenous *APUM9* lies under the control of components of the TGS (MOM1 and NRPE1) (Yokthongwattana et al., 2010). It was also shown to be synergistically regulated by chromatin remodellers DDM1 and DRD1 (Zemach et al., 2013). Activation of *APUM9* expression in the double mutants of *mom1nrpe1* and *ddm1drd1* was shown to correlate with the loss of DNA methylation in the promoter region of the gene (Yokthongwattana et al., 2010; Zemach et al., 2013). Therefore, it was initially assumed that TGS may play the main role in silencing the GFP transgene in the silex.

The silex transgenic line displays a highly tissue specific GFP expression pattern (in a single cell layer in siliques) (Figure 2.1C). As the endogenous *APUM9* was reported to be expressed in the leaf vasculature and young leaves (Abbasi et al., 2011), specific pattern of GFP expression in silex may indicate that there are distinct mechanisms, acting in silencing the transgene in different tissues.

Supporting this suggestion, the release of GFP expression with a stochastic pattern on cotyledons of plants treated with Zebularine indicated that DNA methylation indeed plays role in silencing of the transgene in the silex line (Figure 2.2A). The release of GFP expression only in the stem of *nrpe1* plants indicates that loss of DNA methylation was sufficient to release silencing of the transgene in this tissue, but not in the others (Figure 2.2 B, C). At the same time, the eFP browser (Winter et al., 2007) and proNRPE1::GUS line (created by E. Hristova and not presented in this thesis) indicate that NRPE1 is expressed in tissues of seedlings and young plants as well as in stems and inflorescences of adult plants.

Strong release of GFP expression in young leaves, vasculature of adult leaves, stems and inflorescences of *ago1* plants, indicates that miRNA biogenesis and ta-siRNA production pathways (Figure 1.6, 1.7) may play role in silencing the GFP transgene (Allen et al., 2005; Bohmert, 1998; Fagard et al., 2000) (Figure 2.3). Notably, the observed pattern of GFP release overlaps with the pattern of AGO1 expression (eFP browser, Winter et al., 2007). The *se* mutant had a much weaker effect and released GFP expression only in young leaves (Figure 2.3). Such difference in intensity and GFP expression patterns observed in *ago1* plants and *se* plants, can possibly be explained by that *se-1* mutant, used for this study, is a weak allele of the

mutant. Null mutants of *SE* in *Arabidopsis* were shown to be lethal (Lobbes, D. et al., 2006; Prigge and Wagner, 2001). In addition to this, it has been previously reported that in plants, AGO1-mediated silencing can lead to DNA methylation and to changes in the chromatin structure (Baumberger and Baulcombe, 2005; Chellappan et al., 2010; Dunoyer et al., 2010; Law and Jacobsen, 2010). Thus, we conclude that AGO1 contributes to silencing of the GFP transgene in silex, but the mechanisms remain yet unknown. It may act in biogenesis of a miRNA, which would further target the transgene triggering its silencing. However, this is unprobable, as none of known plant miRNAs (http://www.mirbase.org) displayed high sequence complementarity with the silex transgene, which would be required for such action. Also, as strong release of GFP silencing was observed in *sgs3* and *dcl4* plants, it is more likely that the ta-siRNA pathway, controlled by these components and AGO1, is also involved silencing the GFP transgene (Figure 2.3). This suggests that ta-siRNAs derived from an unknown locus, can contribute to the silencing of the GFP transgene in silex. Production of these ta-siRNAs may be triggered by a miRNA.

On the other side, as previously reported, insertion of a transgene can lead to production of siRNAs, which further guide its silencing by PTGS and /or TGS (Figure 1.1) (Fagard and Vaucheret, 2000; Ingelbrecht et al., 1994; Daxinger et al., 2009). Moreover, it was demonstrated that this process does not require active transcription of the transgene or homology to sequences in the plant genome (Canto et al., 2002). Therefore, it is possible that the GFP transgene in silex itself triggers the production of siRNAs, that could further lead to its silencing by TGS and/or PTGS.

Mutation in DCL4 triggered strong release of GFP expression resulting in a broad GFP signal in adult leaves (covering full leaf plate) and flowers (Figure 2.4, 2.5). In addition, the GFP expression pattern correlates well with the pattern of *DCL4* expression (eFP browser, Winter et al., 2007). Therefore, it can be suggested that the ta-siRNA pathway and possibly PTGS are acting in concert to suppress GFP expression in these tissues.

Similarity of patterns of GFP expression in *ago1* and *dcl4* mutants suggests that PTGS has impact on silencing of GFP transgene in silex line in all these tissues, and dominating over impact of TGS in flowers and adult leaves (Figure 2.6). Interestingly, plants of *nrpe1*, *ago1* and *dcl4* exhibited release of GFP expression in lower parts of stems. It suggests that silencing of GFP transgene in this area may be

achieved via both, TGS and PTGS, but loss of function of at least one of them is already sufficient for release of GFP expression (Figure 2.6). Or conversely our data may implicate that AGO1 and DCL4 play yet unknown roles in TGS.

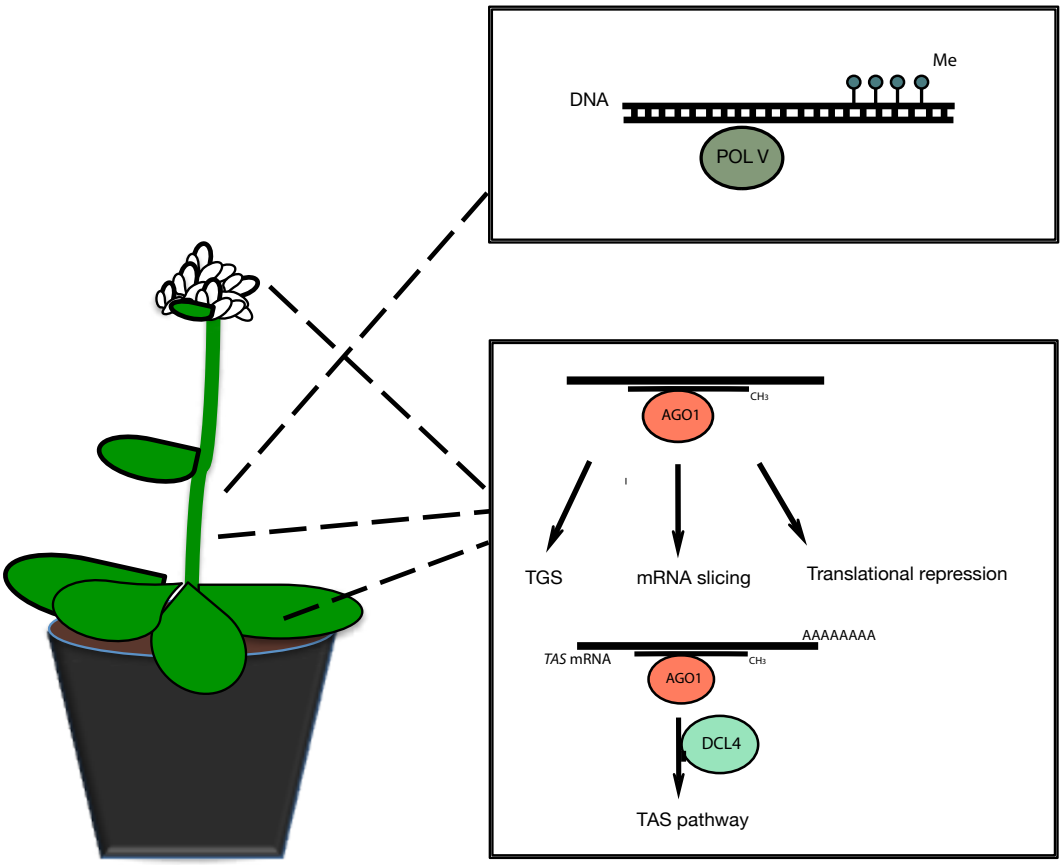


Figure 2.6 Complex mechanisms, involved in silencing the GFP transgene in the silex line

Model, schematically representing the impact of factors from the TGS (PolV) and PTGS (SE, AGO1, SGS3, DCL4) pathways on the expression of the transgene in the silex line. Our results show that mutations in components of PTGS lead to release of GFP expression in leaves, stems and inflorescences, whereas *nrpe1* mutant only exhibited GFP expression in lower part of stems. This suggests that both TGS and PTGS act in repression of the transgene in silex line.

The signal for PTGS through ta-siRNA pathway can derive from a miRNA, targeting the *TAS* precursor RNA. Strong release of GFP expression in *ago1* supports the assumption, that silencing of transgene in silex line could be targeted by secondary small RNAs, deriving from the ta-siRNA pathway and triggered by a miRNA. This hypothesis is strongly supported by resent report of Creasey, K. M. et al. (2014) on miR-directed biogenesis of 21-nucleotide easiRNAs ("epigenetically activated siRNAs"), deriving from the transposon transcripts. The authors demonstrated that this may be the mechanism that specifically targets epigenetically reactivated TEs. The GFP transgene in silex contains a part of the ROMANIAT5 transposon. Therefore it is possible that the transgene might be recognized as a reactivated or newly inserted copy of the TE thus being targeted by a miRNA that triggers the accumulation of easiRNAs and its consequent silencing.

Post-transcriptional gene silencing can be achieved via both RNA slicing or/and translational repression. Unfortunately, it is not yet clear at the moment, which of these mechanisms if at all contributes to silencing of the transgene in the silex line. This remains an objective for further studies.

CONCLUSION

Our results indicated that both, TGS (DNA methylation) and PTGS (miRNA and/or TAS pathways) are involved in silencing the GFP transgene in silex. Impact of the tested factors is limited to certain plant tissues, correlating with predicted pattern of their expression (for AGO1 and DCL4), therefore indicating complex tissue specific regulation by multiple mechanisms (Figure 2.6).

To further investigate the mechanisms, which act in silencing of the transgene in the silex line, it would be important to examine the possibilities of how this process may take place. It needs to be verified, whether the GFP transgene can trigger the accumulation of siRNAs, therefore initiating PTGS or, that it is being targeted by siRNAs derived from a yet unknown *TAS* gene. Also, to investigate the functional connections between the factors, involved in silencing of the GFP transgene, the effects of double and triple mutants of components of the PTGS and TGS on release of GFP expression in silex should be studied. On the other hand, considering that siRNAs might also trigger establishment of the DNA methylation, it would be interesting to test the effect of PTGS factors (also including the double/triple mutants) on DNA methylation in the promoter regions of the transgene and the endogenous *APUM9*.

MATERIALS AND METHODS

Plant material

All plant lines used in this study derive from the Columbia accession. Plants were grown in Sanyo MLR-350 chambers at 24°C with 16 hours light, unless stated different.The silex reporter line was obtained from a collection of The Institute for Genomic Research (J. Craig Venter Institute, line AGRAC-60-1-1) (Xiao et al., 2010). Mutants nrpe1-2 (formerly nrpd1b-2; Pontier et al., 2005), *ago1-27* (Morel et al., 2002), *se-1* (Yang et al., 2006), were used in this study. To study the effect of DCL4 on repression of GFP transgene in silex, the silex line was crossed to *dcl2,3,4* triple mutant (Blevins et al., 2006); the segregating population was then genotyped to identify the plants homozygous by *dcl4* and containing the transgene. The primers, used for genotyping of the lines are listed in Appendix I.

GFP expression in seedlings was observed with Olympus SZX12 binocular (lamp U-RFL-T).

DNA extraction, DNA methylation analysis

Genomic DNA extraction for genotyping was extracted with 500 µl DNA-extraction buffer (100mM Tris-HCl pH8.5, 50mM EDTA pH8, 500 mM NaCl, 10mM ß-mercaptoethanol) with SDS (35µl 20%) followed by incubation for 10 minutes at 65°C. Following the incubation, 130 µl 5M KoAc was add to the samples. After 5 min at 0° C, precipitate was pelleted by centrifugation for 10 min at 13000rpm, 4°C DNA. Supernatant was then precipitated with 640µl of sopropyl alcohol and 60µl 3M NaAc at -20°C. The precipitated pellet was then washed with 70% ethanol and dried at room temperature in DNA 120 SpeedVac (Savant), as described in (Konieczny and Ausubel, 1993) with modifications.

For methylation sensitive PCR, genomic DNA from fresh leaf tissue was isolated using the DNeasy Plant Mini Kit (Qiagen). 50 ng of DNA was then digested with *Dde*I and *Hpa*II restriction endonucleases (NEB) overnight. Digested DNA was PCR-amplified using specific primers for the promoter regions of target genes, designed around the restriction sites. Sequences of corresponding primers are listed in Supplemental the Appendix I.

CHAPTER III On the role of SPLAYED in tissue specific silencing

INTRODUCTION

The specific properties of individual tissues and morphological characteristics of the entire organs are determined by sets of dedicated genes. Expression of such genes needs to be coordinated with developmental and environmental signals and limited to specific tissues (Birnbaum et al., 2003; Breuninger and Lenhard, 2010; Irish, 2010; Li et al., 2008; Schmid et al., 2005; Thain et al., 2002). Therefore, mechanisms that affect the gene expression of the whole plant, act in combination with those that implement the regulation of gene expression in a tissue specific manner (Baubec et al., 2014; Farrona et al., 2011; Manavella et al., 2013).

Several components of mechanisms, regulating genome activity have been identified, revealing complex and connected pathways (Martínez de Alba et al., 2013; Matzke and Mosher, 2014; Numa et al., 2010). However, the described factors affect the general gene expression regulatory pathways, and less is known about the mechanisms that control expression of genes at tissue specific level.

A number of previous studies for identification of factors involved in regulation of gene silencing were based on mutant screens of silenced transgenic lines. These reporter lines mostly contained transgenes with strong constitutive promoters, such as CaMV 35S, that often became silenced (Elmayan and Vaucheret, 1996; Dalmay et al., 2000). Therefore, forward genetic screens were designed to recover mutants, which would display a release in silencing of the transgene. Among the components of the silencing machinery, which were identified in such screens, are the histone deacetylase HDA6 (Murfett et al., 2001) and suppressors of gene silencing SGS2 and SGS3 (Elmayan et al., 1998; Mourrain et al., 2000). However, in order to identify novel factors involved in tissue specific silencing a transgenic line containing an epigenetically silenced tissue specific reporter transgene would be useful.

Therefore, to investigate the contribution of different chromatin regulators to tissue-specific gene silencing in *Arabidopsis,* an approach based on an epigenetically controlled GFP reporter transgene was used. The transgenic line that we termed silex (Xiao et al., 2010) contains an endogenous promoter, that was previously reported to be under complex epigenetic control (Yokthongwattana et al., 2010; Zemach et al.,

2013). Seedlings of silex do not express GFP, however it is detectable in siliques of adult plants (henceforth, the name of the line, Figure 2.1).

Results of our previous studies suggested that silencing of the GFP transgene in silex lies under the control of both, TGS and PTGS (see Chapter II). To identify novel factors defining the tissue specificity of silencing of the transgene, the silex reporter line was mutagenized with ethyl methanesulfonate (EMS) that causes point mutations throughout the genome (Page, 2002). The EMS acts via alkylation of guanine residues (O-6-ethylguanine). During the next DNA replication round thymine residues are incorporated in front of the modified O-6-ethylguanines. Consequently, after the subsequent DNA replication rounds this results in single nucleotide substitutions of G to A and C to T.

Prior to this thesis over 100'000 M2 generation plants (progeny of the self-pollinated EMS treated plants) were screened for the release of GFP expression in seedlings. This allowed retrieving around 150 putative mutants, showing release of GFP expression with various patterns – in young leaves, meristem, leaf margin etc. Assuming that the selected mutant plants may be affected in epigenetic control of gene expression, they were termed *epic*.

This thesis project was concentrated on studying the mutant *epic2,* which exhibited GFP expression in hydathodes and vasculature of leaves.

RESULTS

Tissue specificity of the GFP expression pattern in *epic2*

The recessive *epic2* mutant, identified in the mutant screen, released GFP silencing in hydathodes and veins in young seedlings (Figure 3.1 A) and in veins of fully expanded leaves (Figure 3.1 B). The extent of release of GFP silencing at the mRNA level was measured by quantitative RT-PCR (Figure 3.1 C). Also, the expression of GFP was released in inflorescences of *epic2* (Figure 3.1 D), leading to strong GFP expression in petals of mature flowers (Figure 3.1 E). Western blot analysis using an anti-GFP antibody, confirmed that the green fluorescence observed in *epic2* flowers was indeed linked to the expression of GFP. We detected GFP protein accumulation in inflorescences of the silex line, even though it was not detectable visually (except the valve margin of the fertilized siliques) (Figure 3.1 F).

To better characterize the phenotype of *epic2,* we studied the pattern of GFP expression and morphology of adult mutant plants. To study tissue specificity of the GFP expression pattern in *epic2,* we performed cross sections of leaves and stems. In leaves of *epic2,* GFP expression was associated with cells located around vasculature strands, correlating with location of phloem companion cells (Figure 3.2). Similar correlation was observed on sections of both upper (under inflorescence) and lower (above the rosette) parts of inflorescence stems. There the GFP expression pattern of correlated with partially lignified tissues (Figure 3.3 A, B, 3.4).

To better characterize the phenotype of *epic2*, we performed staining of sections of *epic2* inflorescence stems with toluidine blue, a metachromatic dye that selectively stains acidic tissue components (reviewed in Sridharan and Shankar, 2012). Staining of sections of the lower part of stems with toluidine blue showed visible morphological differences between *epic2* and wild type plants (Figure 3.4). We found that in stems of *epic2* there was a distortion of the normal symmetry in vascular bundles and additional lateral expansion of vascular bundles. Also, sections of *epic2* stems displayed the presence of abnormal differentiation in interfascicular regions (Figure 3.5).

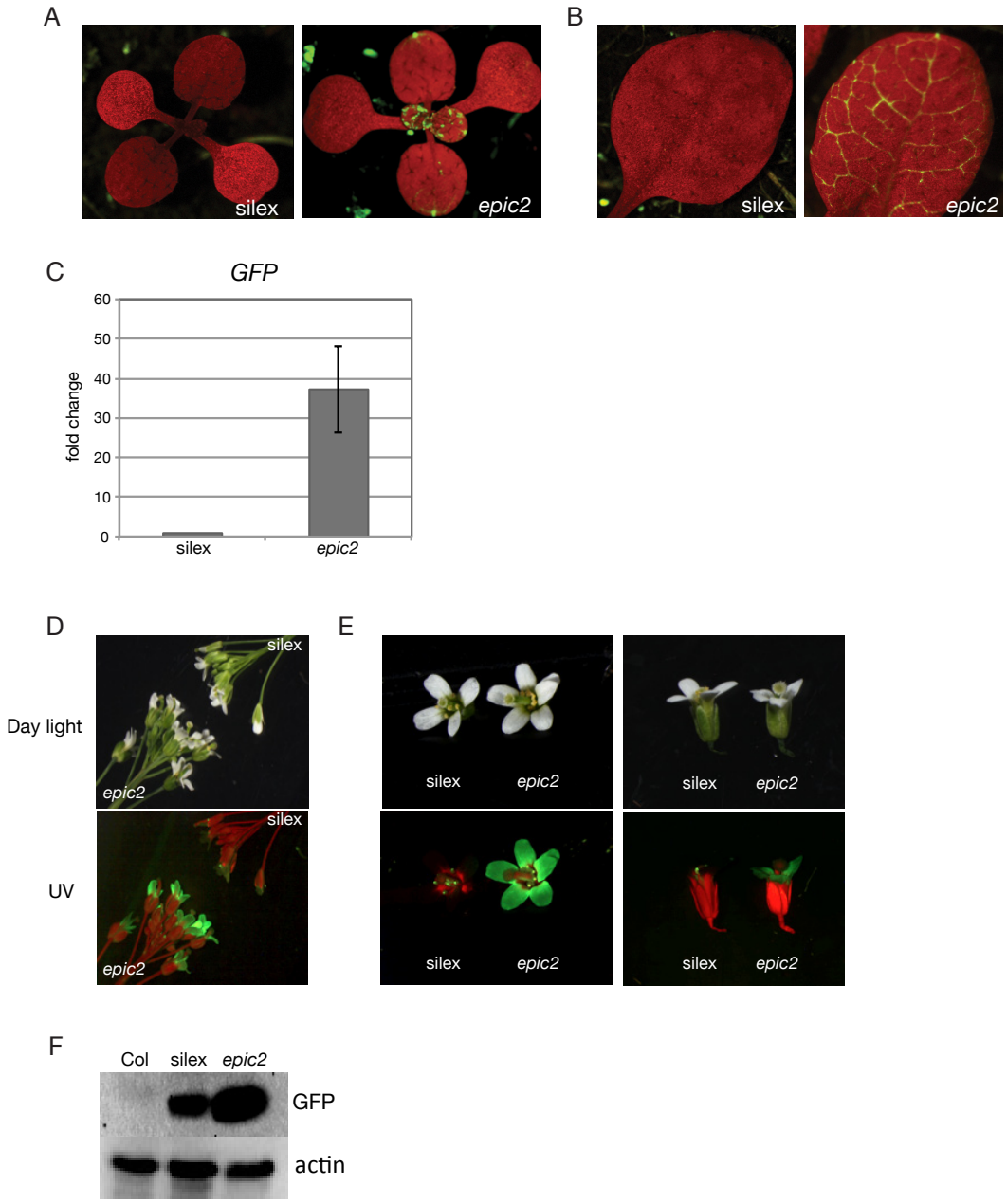


Figure 3.1 Release of GFP expression in tissues of *epic2*

(A) Fluorescence images of silex, and *epic2* seedlings, showing release of GFP expression in hydatodes and young leaves of *epic2*. (B) Fluorescence images of adult leaves of the silex line, and *epic2,* showing release of GFP expression in the vasculature of *epic2*. (C) Quantitative RT-PCR based estimation of release of GFP transcription in seedlings of *epic2* compared to the silex parental line. Error bars show SD of three biological replicates. (D) Day-light and fluorescence images of silex and *epic2* inflorescences, showing the release of GFP expression in inflorescences of *epic2*. (E) Day-light and fluorescence images of individual flowers of the silex line and *epic2*, demonstrating the GFP expression in petals and presence of additional petals in flower of *epic2*. (F) Western blot, showing that GFP is also expressed in inflorescences of the silex line plants, however at a lower level than in inflorescences of *epic2*.

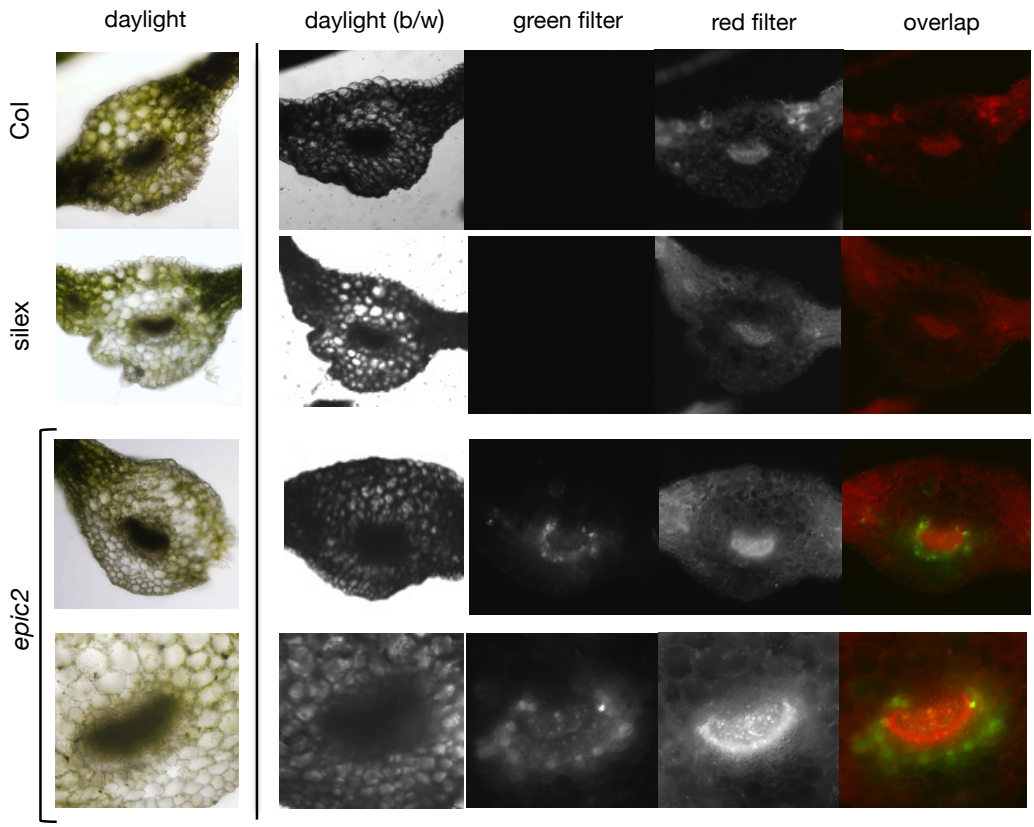


Figure 3.2 Pattern of GFP expression in cross-sections of *epic2* leaves

Day-light (color and black and white) and fluorescence (green and red channels and overlay of the signal) images of hand-cut cross sections of adult leaf plates of wild type plants, silex, and *epic2*. Release of GFP expression was observed in cells at the region of central vascular bundle in leaves of *epic2*, and most likely was associated with the phloem companion cells.

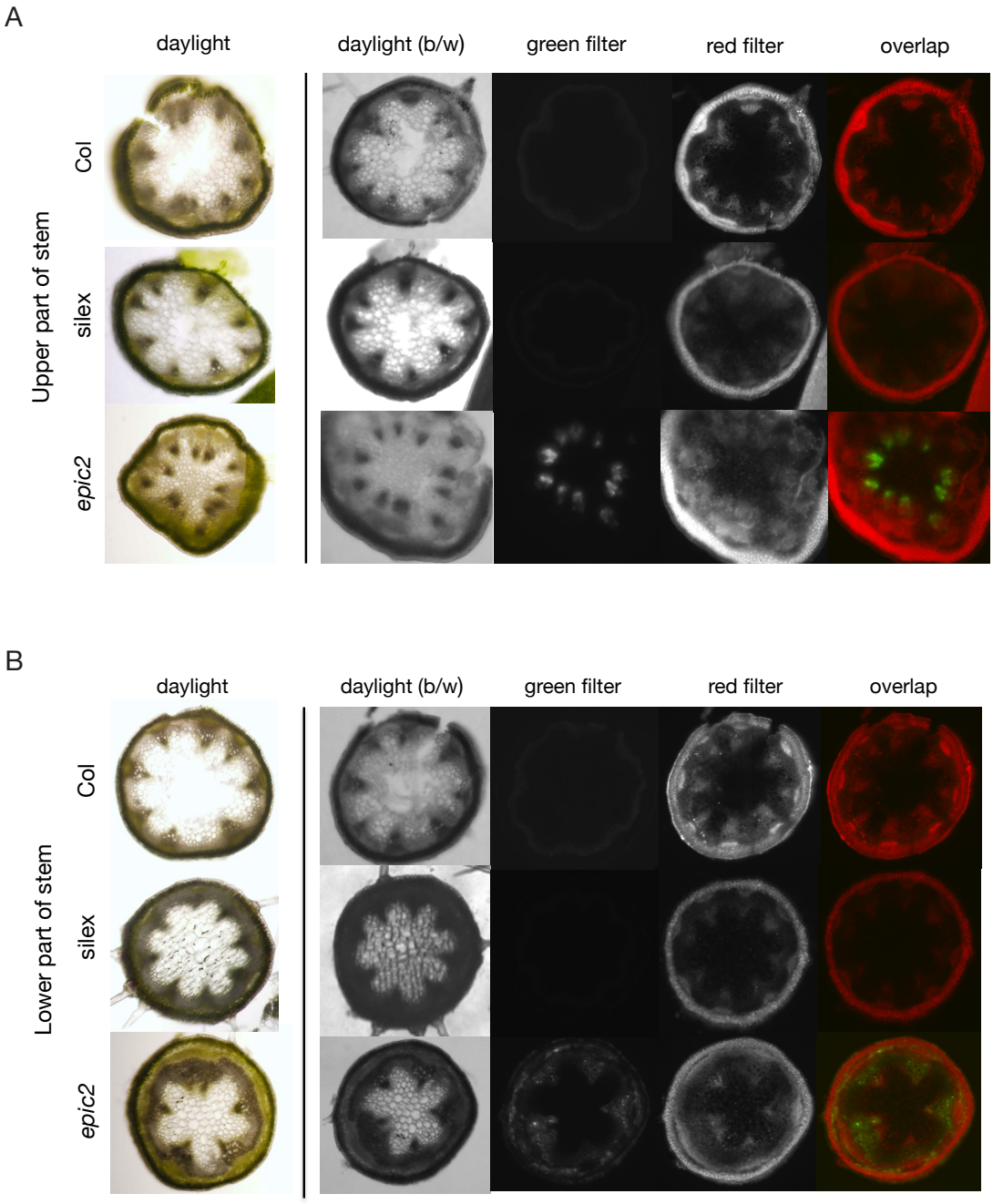


Figure 3.3 Pattern of GFP expression in cross-sections of *epic2* inflorescence stems

(A) Day-light (color and black and white) and fluorescence (green and red channels and overlay of the signal) images of hand-cut cross sections of upper part (2 cm below the inflorescence) of inflorescence stems of the wilt type plants (Col), silex, and *epic2*, showing the release of GFP expression at the regions of vascular bundles. (B) Cross sections of the lower part (2 cm above the rosette) of inflorescence stems of wilt type, silex, and *epic2* plants. *epic2* released GFP expression in the regions of vascular bundles and lignified tissues.

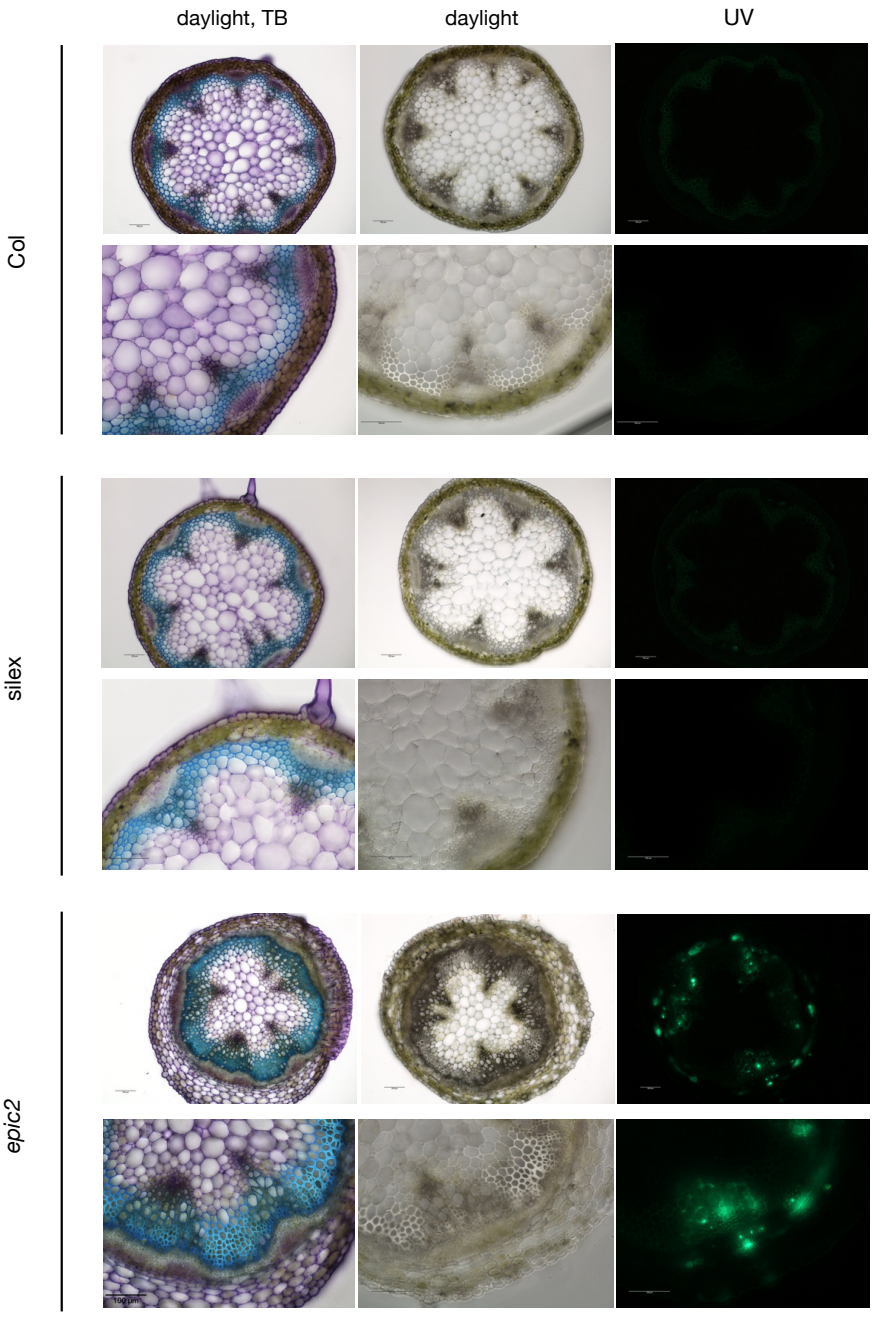


Figure 3.4 Comparison of tuloidine blue stain with GFP expression

(A) Day-light (non-stained and stained with Toluidine Blue) and fluorescence images of cross sections (8µm) from lower parts (2 cm below the inflorescence) of inflorescence stems of wilt type plants (Col), silex, and *epic2*, showing distinct differences in tissues organization of *epic2* stems compared to those of the control lines. Staining with toluidine blue (TB) reveals the xylem and interfascicular cells by their blue-stained cell. The pattern of GFP expression in *epic2* overlaps with the regions of vascular bundles.

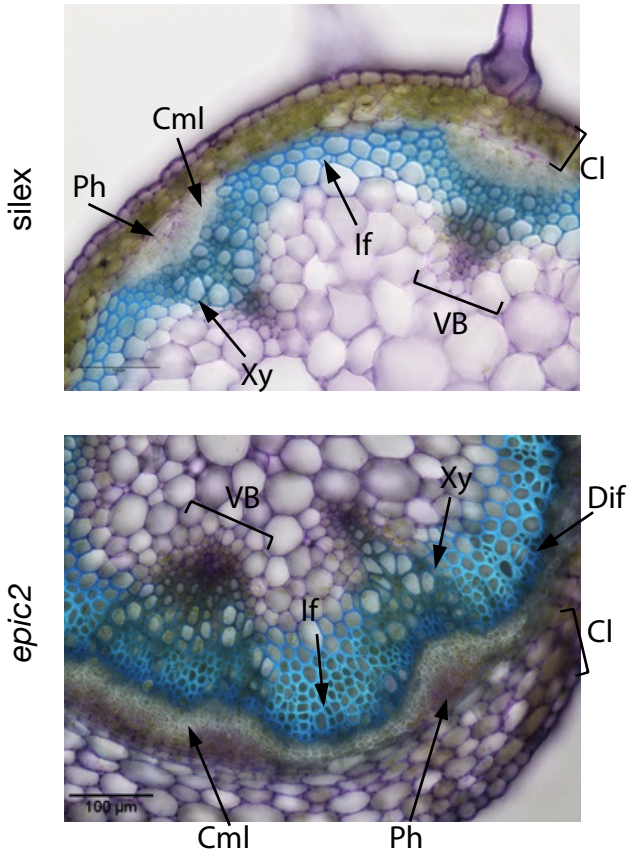


Figure 3.5 Abnormal tissue patterning in *syd-10* inflorescence stems

Day-light images of cross sections (8µm) from lower parts (2 cm below the inflorescence) of inflorescence stems of silex, and *syd-10* plants, stained with toluidine blue. VB - vascular bundle; Ph - phloem; Xy - xylem; If - interfascicular region; Dif - differentiation in the interfascicular region; Cl – cortex layer; Cml – cambium layer. Stems of *syd-10* display overgrowth of the cortex layer in combination unequal development of vascular bundles, thickening of cambium layer and presence of noticeable differentiation in interfascicular regions.

Mapping of *epic2*

In order to map the causal recessive mutation in *epic2,* a combination of whole genome sequencing and classical mapping approaches was used. Frequency of homologous recombination events has been shown to be lower in the regions around the mutations of interest thereby forming regions of genetic linkage (Chang et al., 1988; Eckardt, 2006). In a segregating population of plants resulting from a backcross of a mutant to the parental line followed by selection for a certain phenotype the causal mutation and mutations located closely will be maintained. Unlinked mutations however will segregate normally. In order to map the mutation that leads to the observed release of GFP expression in *epic2*, whole-genome sequencing was performed. DNA was extracted from a pool of 10 GFP positive plants originating from a backcross of *epic2* to the parental silex line. Then, to reveal the regions in the genome, where the recombination frequency is disturbed (regions of linkage), the density of homozygous versus heterozygous SNPs was plotted onto each chromosome (Figure 3.6 A) (James et al., 2013; Meinke, 2003; Uchida et al., 2011). A hot spot for homozygous mutations was found on the lower arm of the chromosome 2 (Figure 3.6 A), therefore indicating the region, where the mutation of interest may be located. The mutations, identified by whole genome sequencing, were further used as genetic markers in further backcrossed populations of *epic2* to map the SNP that caused the GFP expression phenotype. This SNP was located at the last base of exon 22 of *SPLAYED* (*SYD*, *AT2G28290*) within the conserved SWI/SNF domain (Wagner and Meyerowitz, 2002). The mutation in *epic2* is silent with a codon change from AAG to AAA, both coding for a lysine residue. However RT-PCR amplification over the exons 21 to 25 of *SYD* in *epic2,* followed by sequencing of amplified products, showed aberrant splicing variants (Figure 3.6 B and C) present in *epic2* that were not detectable in wild type. By performing genetic complementation tests we recovered a second allele of *epic2*, displaying the same phenotypic characteristics. This second allele carried an amino-acid substitution (S1096F) within the highly conserved helicase domain of SYD (Figure 3.6 D and E). To follow the current nomenclature from now on the two alleles of *epic2* are called *syd-10,* and *syd-11*, respectively (Bezhani et al., 2007) (Table 3.1).

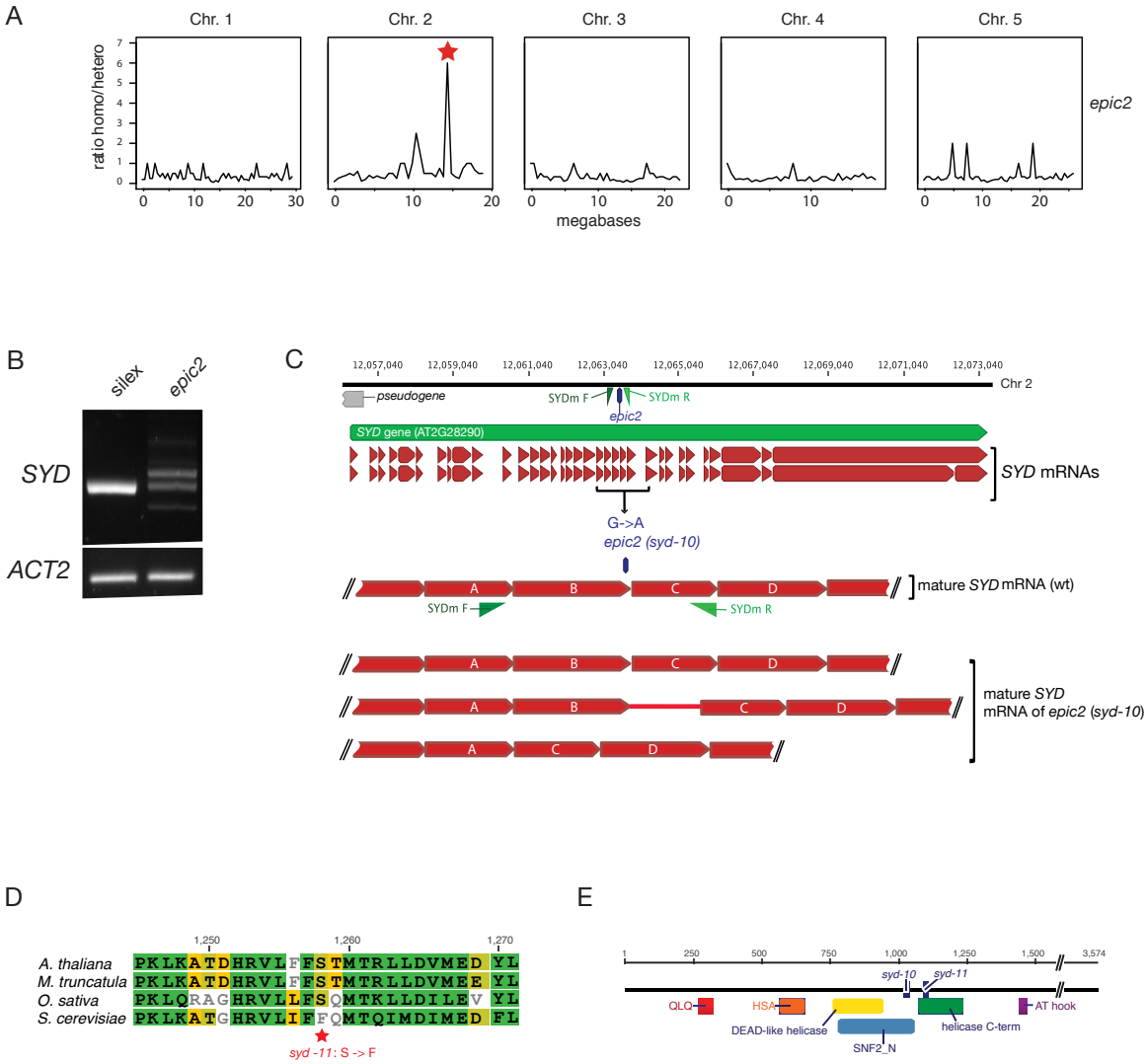


Figure 3.6 Mapping of the *epic2* mutant

(A) The graph representing homozygous versus heterozygous EMS mutation counts in 500 kb windows plotted along the five chromosomes of *Arabidopsis*. A strong linkage disequilibrium (marked with asterisk) was detected at lower arm of chromosome 2. (B) RT-PCR on *SYD* cDNA using primers covering exons 21 to 25. The parental silex line only shows one clear band of the expected size (1170 bp) and *epic2* that carries a mutation at the last base of exon 22 shows multiple splicing variants. The primers, used for amplification are listed in Appendix I. (C) Charaterisation of the splicing variants observed in *epic2* (*syd-10*). The bands shown above were purified from gel and sequenced. In addition to the wild-type clones, clones that maintained the intron between the exons B and C and clones carrying a fusion of the exons A and C, completely lacking exon B were detected. (D) Alignment of aminoascid sequences of helicase domains of the *SYD* orthologs from *A. thaliana, M. truncatula, O. sativa, S. cerevisiae*, illustrating that amino acid change, caused by mutation in *syd-11* is located in domain, highly conserved between these species. (E) Schematic representation of N-terminal part of SYD, including the DNA binding domain, up to AT-hook, showing the location of SNPs in *syd-10* and *syd-11*.

Allele	Lesion	Position (cDNA bp)	Accession	Severity	Described
syd-1	G1152E aa substitution	3572 GGA - GAA	Ler	intermediate	Wagner & Meyerowitz, 2002
syd-2	Q248 -> stop codon	856 CAG - TAG	Ler	null	Wagner & Meyerowitz, 2002
syd-3	A1219V aa substitution	3596 GCT - GTT	Ler	weak	Wagner & Meyerowitz, 2002
syd-4	S912F aa substitution	2792 TCC - TTC	Ler	weak	Wagner & Meyerowitz, 2002
syd-5	SALK_023209 T-DNA insertion	1721	Columbia	null	Alonso et al., 2003
syd-9	SALK_13257 T-DNA insertion	10579	Columbia	strong	Su et al., 2006
syd-10	G -> A substitution	3086	Columbia	strong	This study
syd-11	S1096F aa substitution	3287	Columbia	strong	This study

Table 3.1 List of known alleles of *syd*

The table represents the list of previously reported alleles of the *syd* mutant, and includes 2 alleles (*syd-10* and *syd-11*), recovered from the mutant screen of the silex line and described in this study.

SPLAYED (SYD) is one of the core SWI/SNF chromatin remodeling ATPases of *Arabidopsis* (Flaus et al., 2006; Jerzmanowski, 2007; Wagner and Meyerowitz, 2002). It has been reported to play an important role in plant development (Kwon et al., 2005; Kwon et al., 2006) and in controlling floral organ identity (Wu et al., 2012).

However, so far a role for SYD in gene silencing has not yet been proposed and it is the first time that *syd* was recovered in a mutant screen for factors involved in silencing.

Chromatin remodeler *SPLAYED* is required for silencing the silex transgene in veins

To test whether the *SYD* expression pattern correlated with the tissues in which release of GFP silencing was observed in *syd-10*, a *SYD::GUS* reporter line was created.

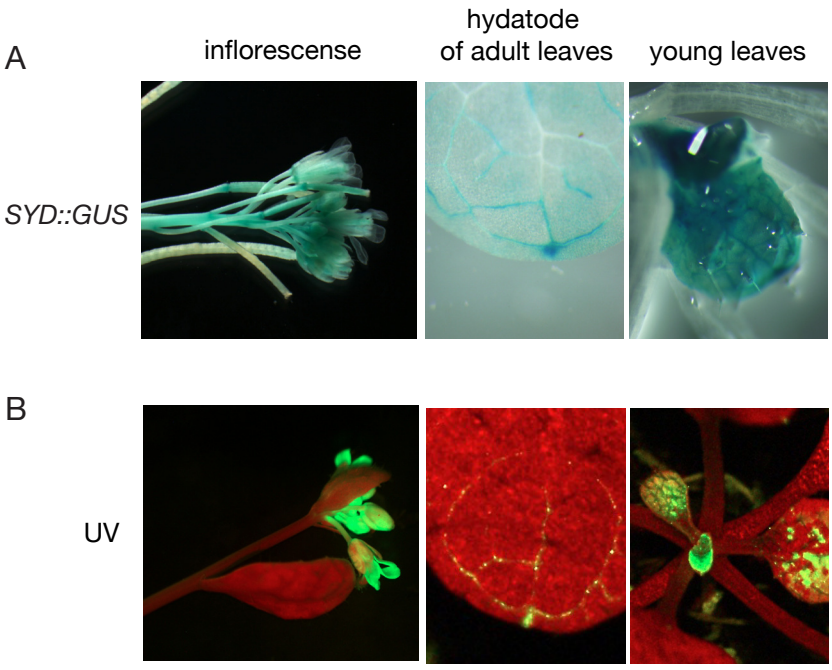


Figure 3.7 Overlap in patterns of *SYD* expression and release of GFP silencing

(A) GUS stained inflorescence, adult and young leaves of representative *SYD::GUS* reporter plants showing the strong expression of *SYD* in inflorescences, hydathode and vasculature of an adult leaf and tissues of young leaves. (B) Fluorescence images of inflorescence, adult and young leaves of *syd-10*, showing the release of GFP silencing in inflorescence tissues and also in vasculature of adult and young leaves.

For this a 2.4kb fragment of the genomic region upstream of the ATG was amplified with specific primers (listed in Appendix I) and cloned into pCAMBIA1304 binary vector (GenBank: AF234300.1). GUS staining was observed specifically in young leaves, hydathodes and vasculature of adult leaves and inflorescences of plants, containing the construct (Figure 3.7 A). This pattern overlaps well with the observed GFP expression pattern in *syd-10* (Figure 3.7 B), therefore, indicating that pattern of release of GFP expression in *syd-10* might be defined by tissue-specificity of *SYD* expression.

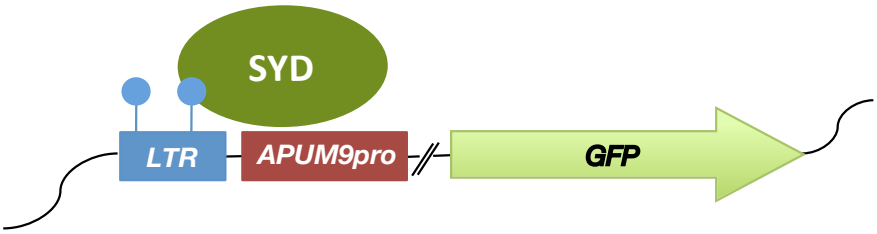


Figure 3.8 Model of direct interaction of SYD with regulatory region of transgene

The proposed model, illustrating SYD silencing GFP expression in the vasculature and inflorescences by directly interacting with the regulatory region of the GFP transgene in silex.

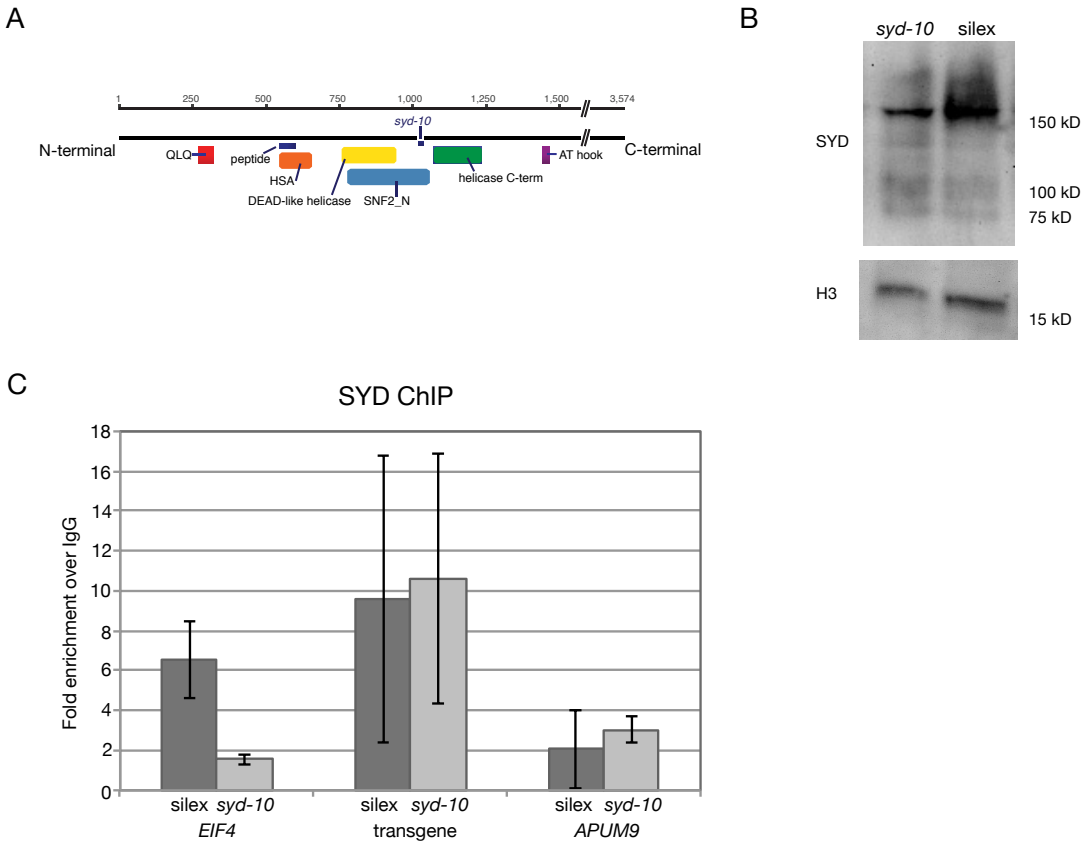


Figure 3.9 Interaction of SYD with promoter regions of the GFP transgene and endogenous *APUM9*

(A) Schematic representation of N-terminal part of SYD, indicating location of sequence of the designed peptide within the HSA domain. (B) Western bolt analysis of nuclear extracts prepared from inflorescence tissues of silex and *syd-10*. Antibodies against N-terminal domain of SYD (Figure 3.9A), and H3 (anti-histone H3; bottom) were used. (C) Anti-SYD ChIP qPCR on material from inflorescences of silex and *syd-10*. Enrichment for SYD in the tested regions of the GFP transgene and *APUM9* was calculated as a fold difference over that, obtained in precipitation with an unspecific antibody (IgG). The primers are listed in Appendix I. Enrichment for SYD in promoter region of the *Eukaryotic Translation Initiation Factor 4A-1* (*EIF4A*), was used as a negative control (Kwon et al., 2005). The error bars show SD of three biological replicates.

The overlap in GFP and SYD expression patterns indicated that SYD might influence transgene expression by directly interacting with its regulatory region (Figure 3.8). Therefore, in order to test this hypothesis, we performed a chromatin immunoprecipitation using a SYD-specific antibody (Figure 3.9 A, B), followed by quantitative PCR amplification of pulled-down DNA. The enrichment for SYD in the tested regions was calculated as fold change over that, obtained from a pull down with unspecific antibody. *EUKARYOTIC TRANSLATION INITIATION FACTOR 4A-1* (*EIF4A*) was used as a negative control (Kwon et al., 2005). The results for SYD in the region of the transgene did not give a conclusive answer, and no significant binding of SYD was detected at the endogenous *APUM9* locus (Figure 3.9 C). Therefore, we envisaged the possibility of an indirect function of SYD in silencing of the GFP transgene, happening through a downstream target of SYD (Figure 3.10).

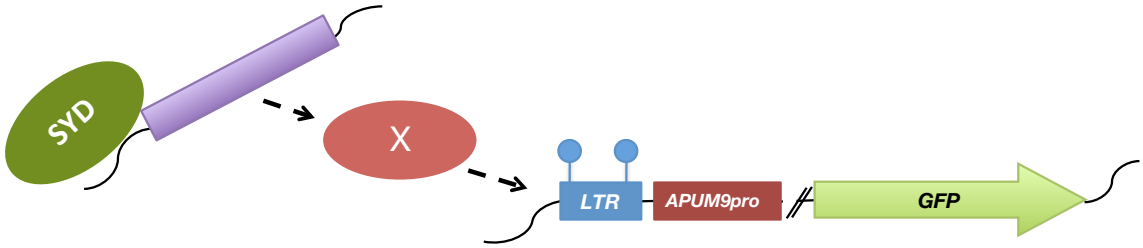


Figure 3.10 Model of indirect regulation of the silex transgene silencing by SYD

The model shows possible indirect connection between SYD and establishment of silencing at the GFP transgene in silex. Such connection may take place through a downstream target of SYD, which may further act in silencing the transgene.

Miss-regulation of the miR156 pathway in *syd-10*

In plants, miRNAs have been demonstrated to regulate multiple developmental processes, affecting the plant phenotype (Pulido and Laufs, 2010; Willmann et al., 2011; Wu, 2013; Yamaguchi and Abe, 2012). In the study on the regulation of anthocyanin biosynthesis in *Arabidopsis,* Gou et al., 2011 reported that an elevated level of anthocyanines accumulated in stems of plants overexpressing miR156 and, consequently, possessing decreased mRNA levels of miRNA156-targeted *SPL* genes.

As the pleiotropic phenotype of *syd-10* also includes red coloring on the top of the inflorescence stem (Figure 3.11 A, B), we first tested whether this effect was indeed associated with accumulation of anthocyanines in these tissues. Measurement of the total fraction of anthocyanines isolated from upper parts of stems was performed as described in Mancinelli et al. 1991 with modifications (the detailed description of the procedure is provided in Materials and methods). The obtained results confirmed that the amount of anthocyanines in *syd-10* was indeed strongly elevated compared to silex parental line (Figure 3.11 C, D).

To better characterise *syd-10* and in order to identify new endogenous targets of SYD, we assessed the transcriptome of 17 days old plantlets by performing RNA-seq on three biological replicates. For this, total RNA was extracted with Ambion mirVana kit (Life technologies) from areal parts of seedlings of *syd-10* and the silex line. The alignment of sequencing reads and quantification of gene expression was performed following the Tuxedo protocol (Trapnell et al., 2012).

The transcriptome revealed a number of genes involved in anthocyanin biosynthesis to be miss-regulated in *syd-10* (Figure 3.11 E). Accumulation of anthocyanines has been reported to be regulated by multiple mechanisms (Deikman and Hammer, 1995; Liu et al., 2013; Misyura et al., 2012; Shin et al., 2013), including the negative regulation by the SPL9 transcription factor, a target of miR156, mentioned previously (Gou et al., 2011). Supporting this suggestion, results of transcription profiling also revealed that several *SPL* genes were miss-regulated in seedlings of *syd-10* (Figure 3.11 F). Thus, it was a first indication that the miR156 pathway might be miss-regulated in s*yd-10.*

As the accumulation of anthocyanines is negatively regulated by the SPL transcription factors (Gou et al., 2011), we next tested expression of the miR156-targeted *SPL3* and *SPL9* in upper parts of *syd-10* stems. Our results showed that expression of both genes in this tissue was down-regulated in *syd-10* (Figure 3.11 G). As mRNA levels of *SPL3* and *SPL9* depend on miR156, we then checked abundance of miR156 and miR172 in upper parts of stems of *syd-10* by northern blot. Interestingly, although down-regulation of mRNA levels of the *SPL3* and *SPL9* indicated possible elevation in miR156 abundance, we observed that level of miR156 was decreased in stems of *syd-10* (Figure 3.11 H).

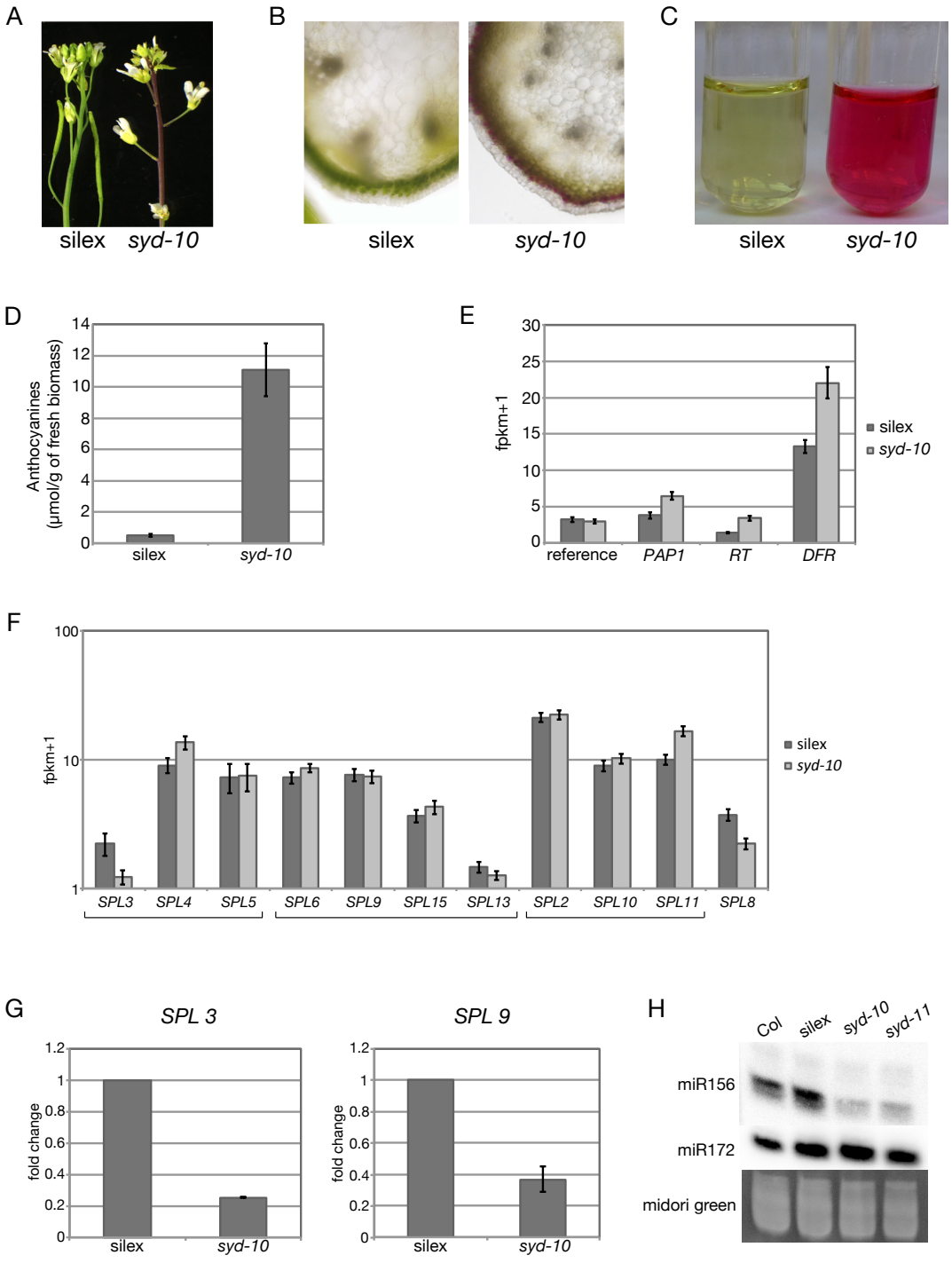


Figure 3.11 Indications of miss-regulation of the miR156 pathway in *syd-10*

(A) Image of inflorescence stems of silex and *syd-10,* showing dark red pigmentation of *syd-10* stem under the inflorescence. (B) Hand-cut cross-sections of upper parts (2 cm below the inflorescence) of stems of silex and *syd-10*. Anthocyanines accumulated in the sub-epidermal layer of cells in upper part of *syd-10* stem. (C) Glass tubes, containing extracted fraction of anthocyanines from the inflorescence stems of silex (left), and *syd-10* (right). (D) Quantification of anthocyaines (µmol/g of fresh

biomass), extracted from upper parts of stems of silex and *syd-10*. Error bars represent standard deviation of 6 replicates. (E) RNA-seq data for silex (dark grey) and *syd-10* (light grey), showing expression levels of genes playing important roles in anthocyanines biosynthesis: *PAP1* (AT1G56650), *RT* (AT4G27570), *DFR* (AT5G42800) and a reference gene (AT1G62930). Error bars represent standard deviation of 3 biological replicates. FPKM stands for "fragments per kilobase of transcript per million mapped reads". (F) RNA-seq data representing expression levels of miR156 targeted *SPL* genes in seedlings of silex and *syd-10.* The represented *SPL* genes are grouped following the classification, suggested by Xing et al., 2011 and presented on Figure 1.8. As above, the error bars represent standard deviation of 3 biological replicates. FPKM stands for "fragments per kilobase of transcript per million mapped reads". (G) Real-time PCR quantification of mRNA levels of miR156-targeted *SPL3* and *SPL9* in inflorescences of silex and *syd-10*. Error bars show SD of three biological replicates; the primers, used for qRT-PCR quantification are listed in Appendix I. (H) Northern blot, showing abundance of miR156 and miR172 in inflorescences of wild type plants, silex, *syd-10,* and the second allele - *syd-11*. A midori green staining of the gel is provided as a loading control.

Colocalisation of *SYD* and *MIR156* expression

Considering the data presented in Chapter II that a strong release of GFP expression was observed in *ago1* (Hutvagner and Simard, 2008), and that the miR156 level was decreased in inflorescence and stems of *syd-10*, we then studied the possibility of miR156 to be involved in silencing the transgene in the silex line.

We first checked, whether the expression patterns of *SYD* and *MIR156* overlap with the GFP expression pattern observed in *syd-10*. For this a non-radioactive *in situ* hybridization was performed with a probe, designed for an N-terminal part of SYD (as described in Wagner and Meyerowitz, 2002) and an LNA probe against miR156 (EXIQON). The obtained results of *in situ* hybridization and fluorescence images of silex and *syd-10* inflorescences (Figure 3.12 A) indicated the presence of an overlap in expression patterns for *SYD, MIR156* (Figure 3.12 A) and GFP (Figure 3.12 B).

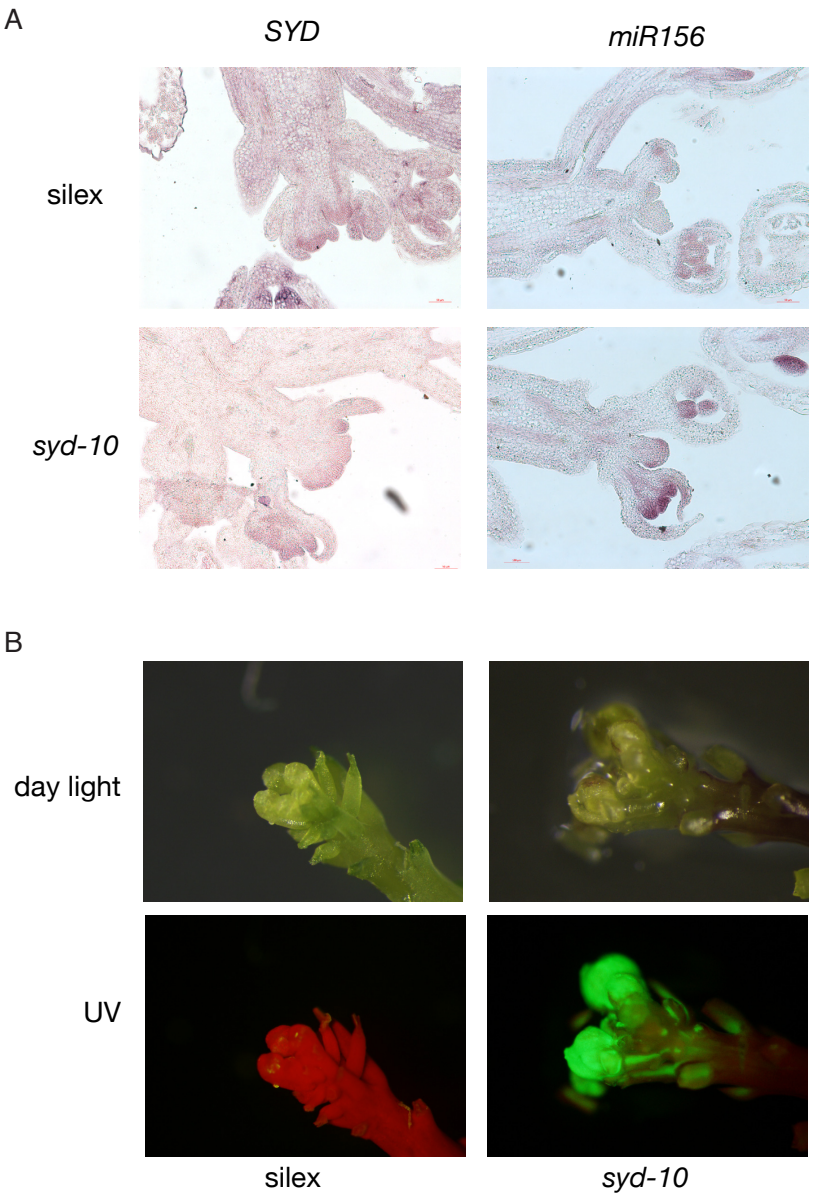


Figure 3.12 Co-localisation of *SYD* and *MIR156* expression with the release of GFP silencing in inflorescences of silex and *syd-10*

(A) Expression of *SYD* (left) and miR156 (right) in cross-sections of silex and *syd-10* inflorescences, visualized by *in situ* hybridization. (B) Day-light (top) and fluorescent (bottom) images of silex and *syd-10* inflorescences, showing release of GFP expression in emerging inflorescences in *syd-10*.

Impact of miR156 on silencing of the GFP transgene

Assuming that miR156 may be in some way involved in silencing of GFP transgene, we then checked whether overexpression or down-regulation of *MIR156* can influence GFP silencing in silex. For this purpose we crossed silex to the miR156 overexpressing line *35S::miR156* (Wu, 2006) and the line *35S::MIM156* which has decreased level of miR156 due to the presence of miR156 mimicry construct (Franco-Zorrilla et al., 2007). No GFP release was observed in silex plants overexpressing miR156. However in the seedlings of plants, containing *35S::MIM156*, GFP expression was observed in young leaves (Figure 3.13), therefore indicating that decrease in level of miR156 can lead to release of GFP expression.

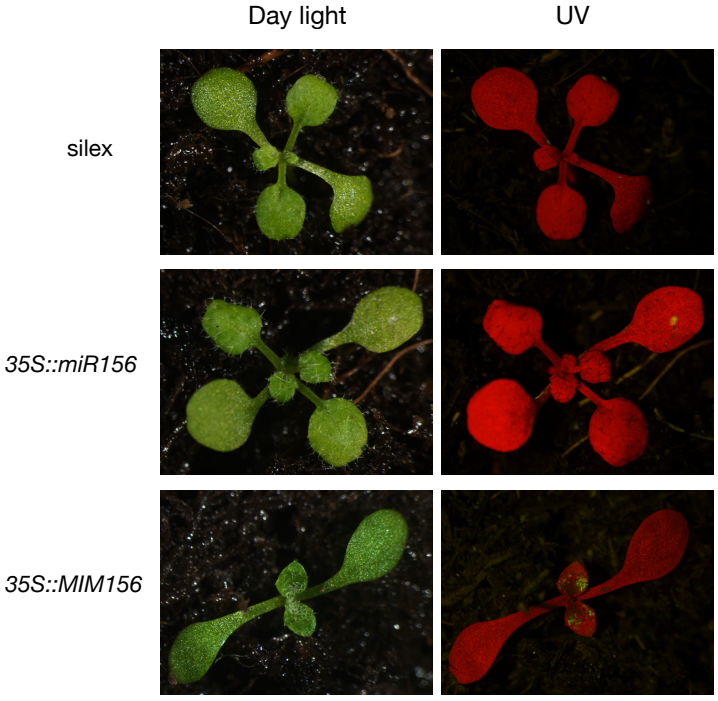


Figure 3.13 Effect of changes in miR156 level on silencing of GFP expression in the silex line

Day-light (left) and fluorescence (right) images of silex plants, plants of *35S::miR156* and *35S::MIM156*, containing the silex GFP transgene. Release of GFP expression observed in young leaves of *35S::MIM156* plants.

MIR156 as a direct target of SYD

Next, we investigated the possibility of SYD acting directly to influence transcription of *MIR156* precursors by binding to the promoters of *MIR156* genes. In order to check the occupancy of SYD in promoter regions of selected genes, we performed a chromatin pull-down with a SYD-specific antibody, followed by q-PCR amplification of DNA (as described before) from promoter regions of *MIR156* genes. The enrichment in tested regions was calculated as a fold difference over that, obtained in pull-down with an unspecific antibody (IgG). *EIF4* was used as a negative control (Kwon et al., 2005). A significant enrichment for SYD was observed in the promoter regions of *MIR156A* and *MIR156C* genes (Figure 3.14), indicating that SYD binds to these regions.

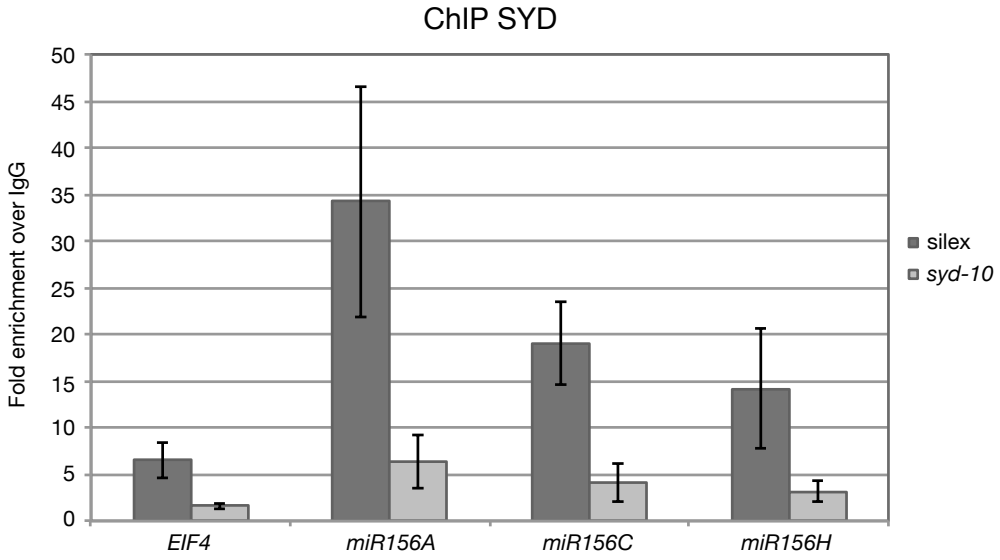


Figure 3.14 Direct interaction of SYD with promoter regions of *MIRNA156*.

Results of ChIP qPCR with anti-SYD antibody, performed on material from silex and *syd-10* inflorescences. Enrichment for SYD in the tested regions of *MIR156A* (AT2G25095), *MIR156C* (AT4G31877), *MIR156H* (AT5G55835) was calculated as a fold difference over that, obtained in precipitation with an unspecific antibody (IgG). The primers, used for quantification are listed in Appendix I. Enrichment for SYD in promoter region of *EIF4A* was used as a negative control (Kwon et al., 2005). The error bars show SD of three biological replicates.

DISCUSSION

In order to uncover new epigenetic factors, controlling tissue specific gene expression, the EMS mutagenesis of the transgenic GFP reporter line (Xiao et al., 2010) was previously performed. This study was focused on characterization of the mutant *epic2*, which was recovered in the screen.

Strong release of GFP expression was observed in vasculature of leaves and stems (Figure 3.1 A, B, 3.2, 3.3), and inflorescence tissues (Figure 3.1 D, E) of *epic2*. The causal mutation in *epic2* was identified to be located within the SWI/SNF domain of the chromatin remodeler SYD (Wagner and Meyerowitz, 2002), leading to production of aberrant splicing variants (Figure 3.6 B and C). Therefore, the *epic2* mutant was further termed *syd-10*.

It was an intriguing discovery, as, although role of SYD in regulation of embryo development and flowering has been reported (Kwon et al., 2005; Kwon et al., 2006; Wagner and Meyerowitz, 2002; Wu et al., 2012), little was known about its function in gene silencing. However, a recent report suggested that the components of chromatin remodeling complex, including SWI3B, SWI3C, SWI3D, BRM and SYD may affect expression of targets of RNA-mediated transcriptional silencing (Zhu et al., 2013). Another indication that SYD might have yet unknown functions in gene silencing was that a number of *Arabidopsis* SWI/SNF ATPases have been previously reported as components of silencing machinery. Among them are DDM1 (SNF2-like, Lsh subfamily), DRD1 (Rad54-like, DRD-like subfamily), ATRX (Rad54-like, ATRX subfamily) and MOM (contains the region with sequence similarity to the ATPase domain of the SWI/SNF ATPases), that are structurally different from SYD, however still belong to the same SWI/SNF superfamily (Amedeo, P. et al., 2000; Higgs et al., 2000; Kanno et al., 2005; Knizewski et al., 2008; Verbsky and Richards, 2001; Richards et al., 1999; Plant Chromatin Database http:/www.chromdb.org; http://www.snf2.net). Notably, it was recently reported that DDM1 and DRD1 also contribute to regulation of endogenous *APUM9* expression (Zemach et al., 2013).

We observed that release of GFP expression in inflorescence stems of *syd-10* is mainly co-localized within regions of lignified tissues and vascular bundles. Also, inflorescence stems of *syd-10* had strong morphological changes compared to the parental silex line and wild-type plants. Observed phenotypic differences include

asymmetry in location and decrease in average quantity of vascular bundles, overgrowth of lignified tissues, differentiation in interfascicular regions and thickening of cortex (Figure 3.4, 3.5). Such affects on morphology of inflorescence stems have so far not yet been described for *syd* mutant.

However, a number of previously characterized mutants have been reported to exhibit alterations of normal stem morphology. Presence of an unusual cambial activity and altered vascular patterning has been shown for *soc1ful* double mutant (Lens et al., 2012). Mutant of WOX4 has severe defects in fascicular, as well as interfascicular, cambial growth (Suer et al., 2011). Also, similar, but stronger phenotype of inflorescence stems was previously reported for *hca* ('high cambial activity') (Pineau et al., 2005), the mutant, impaired in cambial activity and secondary growth throughout the plant body. Ectopic deposition of lignin in stems of *Arabidopsis* has been also reported for plants, overexpressing MYB transcription factors (MYB58, MYB63) (Zhou et al., 2009), and another factor for this family, ALTERED PHLOEM DEVELOPMENT (APL) has been demonstrated to be an important regulator of phloem development (Bonke et al., 2003). Considering the common characteristics between the phenotype of *syd-10* and that of the above mentioned mutants, it is possible that SYD may play a role in tissue pattering within inflorescence stem by controlling expression of factors, regulating the tissue development. However, the transcriptome analysis did not reveal changes in expression for any of the aforementioned genes in *syd-10*. Therefore, it is possible that SYD acts in this process through interaction with yet unknown vascular patterning regulating factor.

The specific release of GFP expression in the vasculature of *syd-10* suggested that SYD plays a role in cell-type specific control of silencing. This was supported by the observation that *SYD* is specifically expressed in the same tissue where release of GFP silencing was observed (Figure 3.7 A, B). At the same time such expression pattern also correlates with the one, previously reported for *APUM9* (Abbasi et al., 2011). Therefore, SYD may contribute to regulation of the endogenous pattern of *APUM9* expression. However, no strong evidence for a direct interaction of SYD with the regulatory region of the transgene was found (Figure 3.9 C), suggesting that the connection between SYD and the transgene in silex is most likely indirect. In this case, specific pattern of release of the GFP expression in *syd-10* can be due to an effect of a downstream factor, targeted by SYD (Figure 3.10). At the same time, the

genes that have been previously reported as targets of SYD (*WUS, AG, STM, AP3*) are known to function mainly as regulators of development and flower patterning and their role in silencing have not been reported (Bezhani et al., 2007; Hamada, S., et al., 2000; Kwon et al., 2005; 2006; Lohmann, J. U. et al., 2001; Wagner and Meyerowitz, 2002; Wu et al., 2012). Consequently, it was decided to further study the *syd-10* phenotype and investigate new targets of SYD that would be directly involved in silencing of the transgene.

At a closer observation of *syd-10* phenotype the attention had been brought to red coloring on the top of the inflorescence stems of mutant plants. Caused by accumulation of anthocyanines, a similar effect was reported in association with overexpression of *MIR156* (Wu, 2006) (Figure 3.11 A - E). Together with the evidence of changed mRNA level of some *SPL* genes (Figure 3.11 F, G), targeted by this miRNA indicated that the miR156 pathway might be disturbed in *syd-10*. Unexpectedly, although over-accumulation of anthocyanines and downregulation of miR156-targeted *SPL* genes indicated possible elevation of miR156 level, results of northern blot analysis demonstrated that level of miR156 in upper parts of stems of *syd-10* was decreased compared to silex (Figure 3.11 H). There are some possible explanations to this effect. First, the northern blot allows detection of only mature form of miR156, not providing the information about abundances of its separate isoforms. Therefore, decrease in level of highly expressed isoform would mask an elevation in level of a less abundant one. So, down-regulation of *SPL3* and *SPL9* and further accumulation of anthocyanines could be caused by increased level of one of the miR156 isoforms on a background of down regulation of the other. On the other hand, considering that SYD has been shown to function primary by activation of gene expression (Bezhani et al., 2007; Kwon et al., 2006; Wu et al., 2012), it is then possible that it directly regulates expression of *MIR156* and miR156-targeted *SPL* genes.

Overlap in expression patterns of *SYD* and *MIR156* and detected release of GFP expression within the corresponding area of inflorescence in *syd-10* (Figure 3.12 A, B) suggested the possibility of direct link between these factors. Further on, introgression of *35S::MIM156* construct to silex line lead to release of GFP expression in young leaves (Figure 3.13), therefore supporting the suggestion that miR156 may target silencing of GFP transgene.

However, known targets of miR156 contain a sequence complementary to

that of the miRNA, allowing recognition of mRNA transcript (Ehrenreich, 2008; Gandikota et al., 2007), whereas blasting the miR156 sequence against the sequence of the GFP transgene gave hits with only low complementarity. Therefore, silencing of GFP transgene by miR156 is most probably not happening through direct mRNA degradation of *GAL4/VP16* or *GFP* transcripts.

The possibility of an indirect interaction between miR156 and the GFP transgene is supported by our previous data that demonstrated strong release of GFP silencing in silex plants, containing a mutation in DCL4, an element of the ta-siRNA pathway (presented in Chapter II). Therefore it is possible that miR156 targets a yet unknown RNA transcript that gives rise to siRNAs themselves targeting the GFP transgene. This idea is supported by resent paper of Creasey, K. M. et al., (2014), reporting on miRNA-directed 'epigenetically activated' small interfering RNAs (easiRNAs) specifically targeting the transcripts of reactivated TEs and suggesting that miR156 might be involved in the tissue-specific silencing of transposable elements during different phases of plant development. It is possible that the fragment of *ROMANIAT5*, included in the transgene might be recognized as a reactivated copy of *ROMANIAT5*, thus, triggering the production of siRNAs and consequent silencing of the GFP transgene. A number of siRNAs were found to be present within the region of the *ROMANIAT5 LTR* and the endogenous promoter of *APUM9*. However, it is not yet clear, whether any of them have a direct effect on the expression of the GFP.

To check, whether SYD indeed directly affects transcription of miR156 precursors, we tested if it could interact with regulatory regions of *MIR156* genes. Unfortunately, no data on genome-wide studies of SYD-binding regions has been reported yet. However, previously published reports on ChIP-seq for LFY, an interaction partner of SYD, indicated the representation of LFY at the regions close to the micro-RNA genes (Moyroud, E., 2011). As LFY has been shown to be involved in recruitment of SYD to its target genes (Wu et al., 2012), presence of LFY in proximity of *MIR156* genes further supported our hypothesis.

Results of ChIP quantitative qPCR showed that SYD binds to promoter regions of *MIR156* genes (Figure 3.14). Therefore, indicating that SYD acts upstream of miR156, regulating the expression of its precursor transcripts and thus influencing its ability to target silencing of the GFP transgene in silex (Figure 3.15).

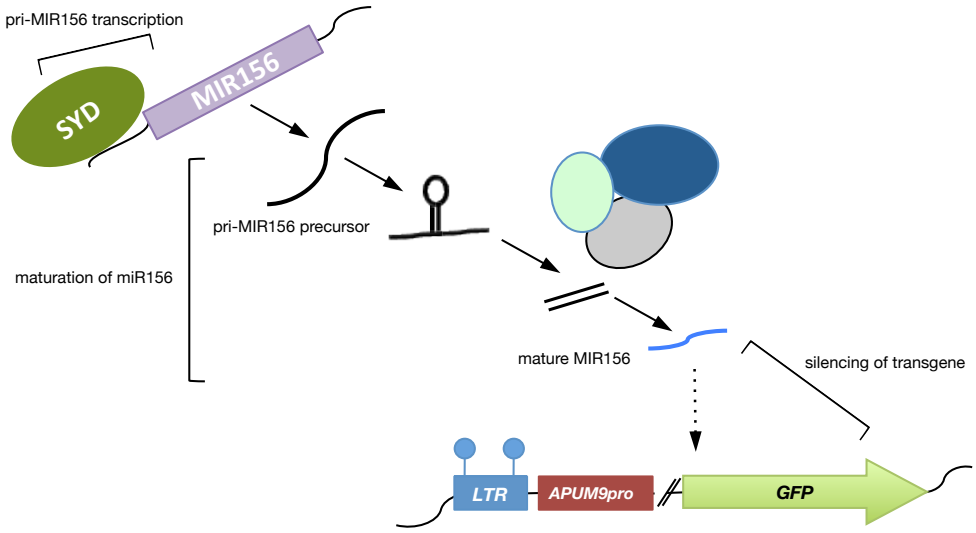


Figure 3.15 SYD may contribute to silencing of the GFP transgene in silex by controlling transcription of *MIR156*

Summarising model, showing the indirect role of SYD in silencing of the GFP transgene in silex. SYD acts as a direct regulator of transcription of several *MIR156* genes. As a result, in *syd-10* the decrease in level of miR156 can be observed. It its turn, mature miR156 indirectly plays role in silencing of the GFP transgene in silex.

CONCLUSIONS

We found that the mutant, recovered in the designed forward genetic mutant screen is affected in SYD chromatin remodeling ATPase. The mapped *syd-10* mutant exhibited GFP expression in vasculature tissues and inflorescences. We showed that mutation in *SYD* leads to miss-regulation of *MIR156* accumulation. Also, we showed that miR156 indirectly contributes to silencing of the GFP transgene in the silex line. Consequently, down-regulation of miR156 in *syd-10* leads to release of silencing from GFP transgene in the tissues, where SYD is expressed. However, further studies are required to identify, whether the connection between miR156 and the GFP transgene is direct or indirect.

MATERIALS AND METHODS

Plant material, mutagenesis and mapping

All plants used in this study come from the Columbia accession. The silex reporter line was obtained from a collection created by The Institute for Genomic Research (J. Craig Venter Institute, line AGRAC-60-1-1) (Xiao et al., 2010). Other lines used in this work were: *35S::miR156* (Wu, 2006) and *35S::MIM156* (Franco-Zorrilla et al., 2007). EMS mutagenesis was carried out as described in Weigel *et al* 2002.

Causal EMS mutations were mapped by whole genome sequencing combined with classical mapping by crossing the mutants with the Wassilewskija accession (WS). Reads were mapped against the reference genome and SNPs called in Geneious (Biomatters Ltd.). Using R SNPs were filtered for EMS mutations (G->A) and zygosity called based on the variant frequency provided by Geneious (>= 80% homozygous mutation, >= 45% and <= 55% heterozygous mutation). Plots were then created by calculating the ratio of the number of homozygous and heterozygous and mutations in a 500 kb window (Figure 3.6 A).

Unless otherwise stated plants were grown in Sanyo MLR-350 chambers at 24°C with 16 hours light.

Splicing analysis of the *SYD* transcript

To check for presence of multiple spicing variants of mutated gene in epic2 mutant, cDNA from epic 2 and silex in the region around mapped mutation was PCR amplified with specific primers (Appendix I), using GoTaq DNA Polymerase (Promega). Obtained PCR products were analized on 4% agarose gel.

Real-time PCR and RNA-seq

Total RNA from 100mg of fresh leaf tissue of Arabidopsis plants was isolated with innuPREP Plant RNA Kit (Analytik Jena). 500 ng of RNA were used for cDNA synthesis (iScript cDNA synthesis kit, Bio-Rad). Expression of target genes was measured by quantitative PCR (qPCR) in a Light-Cycler 480 (Roche), using SYBR Green I Master Mix. Primer sequences are presented in Supplemental Table 1.

Expression results were normalized to *ACT2* (Fig. 2-4) or *UBQ10* (Fig. 6). To check for presence of multiple spicing variants of *SYD* in *epic2*, cDNA from silex and *epic2* was PCR amplified using GoTaq DNA Polymerase (Promega) and PCR products analyzed on a 4% agarose gel. RNA-seq was performed on total RNA extracted using the Ambion mirVana kit (Life technologies) from 17 days old plants (areal parts only) grown on soil. Sequencing reads were then aligned and gene expression quantified following the Tuxedo protocol (Trapnell et al., 2012).

Sectioning and staining of tissues

GFP expression in seedlings was observed with Olympus SZX12 binocular (lamp U-RFL-T).

Hand-sections of adult leaves and stems were performed with a razor-blade, for further microscopy, sections were mounted in 50% glycerol media. GFP fluorescence in sectioned tissues was visualized and pictured with a Nikon eclipse *80i* epifluorescence microscope using green, and red filters. Overlay of pictures, taken with green and red filters was performed with OpenLab software.

For differential staining, fresh tissues were embedded in 4% agar. Sections of 8µm thickness were performed on vibrating microtome 5000mz-2 (EMS). 0,01% Toluidine blue solution was applied on sectioned tissues for 40 seconds, followed by subsequent washes with water. Stained sections were mounted in water and examined with Leica TCS SP2 AOBS microscope.

Extraction of antocyanines

Anthocyanines from plant tissues were extracted as described in Mancinelli et al.1991 with modifications: 150mg of fresh tissue were collected and grinded with liquid nitrogen to a fine powder (20sec 7 times). Obtained powder diluted in 750ul of extraction solution (80%methanol, 5% HCL) and incubated 12h at 4°C in the darkness. The extract was then centrifuged 20 min 14000 rpm and supernatant transferred in a new tube. The light absorption of extract was measured at 530 and 657nm (λ_{max} for anthocyanines and chlorophyl respectively). Concentration of anthocyanines in the tissue is calculated as follow: $A=A_{530}-0.25*A_{657}$, where A_{530} - absorption value for anthocyanines, A_{657} - absorption value for chlorophyll. Final

number (μmol/g of fresh biomass) is a mean value of 6 replicates, each replicate containing material of 5 plants pulled together.

Cloning and transformation of *Arabidopsis*

A fragment of 2.4kb from the region upstream of the ATG of SYD (AT2G28290) was amplified with specific primers (Appendix I) and cloned into pCAMBIA1304 expression vector (GenBank: AF234300.1). Arabidopsis plants were transformed by floral dip method (Clough and Bent, 1998).

GUS staining

Staining for GUS (*GUS*: β-glucuronidase) was performed by fixation in cold acetone (10 minutes) followed by vacuum infiltration with the Staining solution (50mM NaPi, pH7.0, 0.5mM $K_3Fe(CN)_6$, 0.5mM $K_4Fe(CN)_6$, 10mM EDTA, 1mM X-Gluc) and incubated at 37°C overnight. Further tissues were distained by multiple washes with 70% ethanol and stored in 50% glycerol (Jefferson et al., 1987).

DNA extraction

Genomic DNA extraction for genotyping was extracted with 500 µl DNA-extraction buffer (100mM Tris-HCl pH8.5, 50mM EDTA pH8, 500 mM NaCl, 10mM ß-mercaptoethanol) with SDS (35µl 20%) followed by incubation for 10 minutes at 65°C. Following the incubation, 130 µl 5M KoAc was add to the samples. After 5 min at 0° C, precipitate was pelleted by centrifugation for 10 min at 13000rpm, 4°C DNA. Supernatant was then precipitated with 640µl of isopropyl alcohol and 60µl 3M NaOAc at -20°C. The precipitated pellet was then washed with 70% ethanol and dried at room temperature in DNA 120 Speed Vac (Savant), as described in (Konieczny and Ausubel, 1993).

RNA preparation for Northern blot

Total RNA was isolated from 250 – 300 mg of fresh plant material by using the mirVana miRNA Isolation Kit (Ambion). For small RNA blot, 25µg of total RNA (extracted as above) with equal volume of loading buffer (95% formamide, 20mM EDTA pH 8, 0.05% bromophenol blue, 0.05% xylene cyanol: for 10 ml, 9.5 ml

formamide, 0.5 ml 200mM EDTA ph 8) were predenatured at 90° for 2 min and loaded on 15% poly acrylamide gel with 0.5g/ml urea. Gel running conditions were as following: 200 V, 30 mA , 6W. Further, the RNA was transferred to a Hybond N+ membrane in TBE 1x buffer, 10V in cold room overnight. Membrane UV crosslinked with a dose of 1400 joules.

In situ hybridization

Specific patterns of expression of miR156 and *SYD* in inflorescences were studied by non-radioactive in situ hybridization (Carles et al., 2010). An LNA probe for miR156 (G*TGC*TCA*CTC*TCT*TCT*GTCA/3Dig_N), where locked nucleotides are marked with an asterisk. was ordered from EXIQON (http://www.exiqon.com/ls). An anti-sence probe for *SYD* mRNA was designed in the N-terminal region of the gene, as described in (Wagner and Meyerowitz, 2002). Amplified PCR product was ligated into pGEM vector. Anti-sence probe was amplified from T7 promoter with T7 RNA Polymerase (Promega P2075).

Plant material was sampled into vials, containing fixative solution (4% paraformaldehyde in 1×PBS, pH7 with 1ml Triton X-100 and 1 mL of DMSO per 100 ml of solution) and vacuum infiltrated for 40 min (2min+8 min+3x10min) on ice. It was followed by an incubation of samples with a fresh fixation buffer overnight at 4°C with shaking. Sampled tissues were then dehydrated and stained in a graded alcohol series with EosinY, which facilitates sectioning. Ethanol was further gradually replaced with tissue-clearing solution Histoclear (National diagnostics No.HS-202). After full replacement of solution to Histoclear, it was exchanged 3 times (with incubation of at least 2 hours between the changes). Then into vials, containing samples in Histoclear, were placed paraplast embedding wax chips (Paraplast X-TRA, McCormiK Scientific REF 39503002) and incubated overnight at 45°C. Later, samples were placed at 60°C oven and melted paraplast solution was gradually added and replaced every 6 hours (5 – 6 times). Poured into moulds embedded tissues were stored at 4°C. Embedded tissues were sectioned to ribbons of 8µm on rotary microtome (MICROTOM Microm HM355) and placed onto Probe ON Plus microscope slides (Fisher Scientific Cat.No: 22-230-900).

Slides were incubated in 100% Histoclear for de-waxing the and then rehydrated in gradual ethanol series, from 100% to water (exchanging the solution

after 1 min). After last wash with water, slides were treated as following: 0.2 M HCl, for 20 min, Water, for 5 min, proteinase K buffer (100mM TrispH8, 50mM EDTA pH8, Proteinase K 1µg/ml) for 30 min at 37°C, 1xPBS, for 5 min. Then the slides were washed with Glycine buffer - 2mg/ml of glycine in 1xPBS (130mM NaCl, 7mM Na_2HPO_4, 3mM NaH_2PO_4, pH7.0) for 2 min and with 1xPBS for 30 sec. To refix the tissues after Proteinase K treatment, the samples were further incubated with 3.7% formaldehyde in 1xPBS for 20 min. Next, the slides were washed in 1xPBS for 5 min and dehydrated in graded ethanol series (from water to 100% ethanol). Prior to habridization, slides were dried under vacuum and incubated with hybridization buffer, containing 6XSSC (0.3M NaCl, 30mM $Na_3C_6H_5O_7$), 3% SDS, 50% deionized formamide, 0.1mg/ml yeast tRNA, at 55°C for 1 hour (prehybridization). Denatured probe was mixed with warmed hybridization buffer and allied on slides. Hybridization was performed overnight at 55°C.

After hybridization, slides were washed twice with SSC washing buffer (0.2xSSC/0.1%SDS) at 55°C for 10 min, incubated with 10µg/ml RNase in 2xSSC at 37°C for 30 minutes then again two times with SSC washing buffer. After last wash, slides were rinsed with 2xSSC, then with with 1xTBS (0.4M NaCl, 1M Tris-HCl pH7.5) for 5 min at room temperature and placed into Blocking buffer (0.5% Boeringer Blocking reagent in 1xTBS for 1 hour at room temperature). Further, slides were washed with Washing buffer 2 (1xTBS, 0.5%BSA) for 1 min at room temperature and incubated with Anti-DIG AP conjugate (Roche, Ref. 11093274910; 1:500 in Washing buffer 2) for at least 3 hour at room temperature. Next, slides were washed with 1xTBS, 0.5% BSA, 0.1% Triton for 10 minutes at room temperature with shaking. This step was repeated 4 times. Finally, slides were incubated for 15 minutes with Detection buffer (0.1M Tris pH9.6, 0.1M NaCl, 50mM $MgCl_2$). Substrate - 1.6µl of BCPIP (5-Bromo-4-chloro-3-indolyl phosphate, Roche, Ref. 11383221001) and 2.2µl of NBT (Nitroblue tetrazolium, Roche, Ref. 11383213001) per 1 ml of Detection buffer was prepared (with calculation of at least 100µl per slide), and applied to slides, followed by incubation in darkness at room temperature. Intensity of signal was estimated after 24hours of incubation and the detection solution exchanged. Detection reaction was stopped by dipping the slides in water. After that the sample slides were mounted in 50% glycerol.

Western blot

For verification of GFP expression in inflorescences, a crude extract was prepared by adding 100µl of 1xPBS buffer (58mM Na2HPO4, 17mM NaH2PO4, 68mM NaCl, pH7.4) to 100mg of fresh tissue, frozen in liquid nitrogen and grinded. Next it was centrifuged at 13000rpm at 4°C, supernatant was transferred to a fresh tube with equal amount of 2xSDS loading buffer (25% Tris-HCl 0.5M pH6.8, 4% SDS, 3 β-mercaptoethanol, 20% glycerol, bromophenol blue) denatured at 90°C and run on 10% SDS-PAGE gel. The semi-dry transfer was performed at 50mA, 1 hour with Transfer buffer (48 mM Tris, 39 mM glycine, 0,0375 % SDS, 20% methanol). Further the membrane was incubated with Blocking buffer (1% dry milk, 0,4% Tween, in 1xPBS) overnight at 4°C. Primary antibodies anti-GFP (Molecular probes A-11120), anti-Actin (SIGMA A3853), and secondary anti-mouse serum (Sigma A3562) were diluted in Blocking buffer as 1:30000. Following washes were performed with the Washing buffer (1% dry milk, 0.5% BSA, 0,4% Tween, in 1xPBS). CDP-star reagent (NEB #N7001) was used for detection of the signal.

SYD-specific antiserum was generated against peptide sequence (MKEERQRRIRERQKE-C). Anti-SYD antibody was raised in rabbits (EZBiolab). Prior to use the antibody was purified on an antigen column (procedure described in Campbell et al. 1951). Specificity of the antiserum was confirmed by a Western blot on sonicated nuclei fraction. Fraction of nuclei was extracted from 0.5g of fresh Arabidopsis tissue (inflorescences), following the protocol for Chromatin immunoprecipitation (Jaskiewicz et al., 2011) with (Roche, product No 11134515001) and protease inhibitor (Halt Protease Inhibitor Cocktail, EDTA-Free (100X), Product No 78437) and 20mM PMSF. Obtained pellet was sonicated with Bioruptor UCD-200 to fragments of 0.3 – 0.5kb. 40µl of denatured samples with equal volume of 2x SDS loading buffer was run on 7% gel. Further, membranes were hybridizes with SYD antiserum (1:500) and H3 antibody (pAB-060-050, Diagenode) as a loading control, followed by probing with secondary anti-rabbit HRP (1:30 000, Sigma A3687), respectively. The detection was performed with CDP-star reagent (NEB #N7001).

Chromatin immunoprecipitation

For the Chromatin immunoprecipitation (ChIP), plant material was cross-linked in infiltration buffer (13,69g Sucrose, 1ml PMSF 100 mM, 1ml Tris/HCl 1M pH8, 200µl EDTA 0.5M, 2.7 ml formaldehyde 37% per 100ml) in proportion 40ml of buffer per 0.5g of material.

Infiltration performed on ice (3 times, 10 min), then 2ml of 2M Glycine added to stop the fixation and infiltrated for 5 minutes. Later. Samples are washed with 500ml of MQ water, dried on paper and frozen in liquid nitrogen.

Extraction of nuclei was performed as described in Jaskiewicz et al., 2011 and 20mM PMSF. Obtained pellet was sonicated with Bioruptor UCD-200 to fragments of 0.5 – 1kb. 10mg of Chromatin was used for immunoprecipitation with specific antibody on Protein A agarose beads (Roche, product No 11134515001) and protease inhibitor (Halt Protease Inhibitor Cocktail, EDTA-Free (100X), Product No 78437) overnight at 4°C.

Bound fraction wased 3 times with a Washing buffer (25mM Tris/HCl, pH 8.0, 5mM EDTA pH 8.0, protease inhibitor 1x), 3 times with LiCl buffer (250 mM LiCl, 10 mMTris-HCl pH8.0, 1 mM EDTA pH8.0, 0.5% NP-40 (Igepal), 0.5% Na deoxycholte, protease inhibitor 1x), and once with a TE buffer. All washing steps performed at 4°C.

Elution of precipitate performed by adding 100 ul of Glycine elution buffer (0.1M glycine, 0.5M NaCl, 0.05% Tween-20, pH2.8) to the beads pellet. De-crosslinking was performed by adding 2ul of RNAase A 10mg/ml (1hour incubation at 37°C) and 3ul of Proteinase K, 1mg/ml (3 hours, 65°C). Precipitated DNA was extracted with High Pure PCR Cleanup Micro Kit (Roche, Product No 04983912001).

Standard curves for qPCR were built based on amplifications of dilution series from pulled input samples. PCR primers were designed for promoter regions of target genes, sequences of the primers are listed in Appendix I. Binding of SYD protein to a region was estimated by fold difference in percentage of enrichment over input by specific antibody over IgG and compared to that in the proximal region of the *Eukaryotic Translation Initiation Factor 4A-1* (*EIF4A*), which was used as a negative control (Kwon et al., 2005). The error bars represent an error of 3 biological repeats.

CHAPTER IV SPLAYED controls miR156 dependent developmental phase transitions in *Arabidopsis*

SUMMARY

In plants, the evolutionary conserved microRNA miR156 plays important roles in regulating the juvenile-to-adult and vegetative-to-reproductive developmental phase transitions. These phase transitions are controlled by antagonistic activities of miR156 and its target mRNAs encoding several SQUAMOSA PROMOTER BINDING PROTEIN-LIKE (SPL) transcription factors. While miR156 promotes the juvenile phase, SPLs support the adult phase and the competence to flower. As plants develop, flowering is facilitated by a decrease in miR156 levels thereby allowing accumulation SPLs. So far, only a few factors involved in the transcriptional control of *MIR156* genes have been identified.

Spatiotemporal control of gene expression during plant development is determined by complex interactions involving transcription factors and changes in the chromatin structure. SPLAYED (SYD) is a well-characterized SWI2/SNF2 chromatin remodeling ATPase, which has been shown to be an important regulator of flower organ identity and homeotic gene expression.

miR156 and SYD are both known to play important roles in plant development and reproduction. In this study we investigated the interaction between these two developmental regulators. We provide evidence that SYD controls developmental phase changes by directly modulating transcription of several *MIR156* and *SPL* genes in *Arabidopsis*. Based on our findings, we propose that SYD acts in the regulation of miR156-dependent and miR156-independent pathways during plant development.

INTRODUCTION

Growth and development of plants is coupled to major changes that take place during transitions from embryonic to postembryonic, juvenile-to-adult and later from vegetative-to-reproductive phases. Timing of developmental phase transitions involves integration of complex environmental and endogenous signals (Achard, 2006; Poethig, 2003). The factors that have been shown to affect developmental phase transitions include ambient temperature, water, photoperiod, light intensity and quality and, on the other side, hormones (e.g. gibberellins) and carbohydrates (e.g. sugars) (Franklin, 2009; Kim et al., 2004; Koch, 2004; Lambers et al., 2008; Matsoukas, 2014; Ohto et al., 2001; Srikanth and Schmid, 2011; Yu et al., 2012).

The microRNA miR156 is highly conserved in the plant kingdom and has been shown to play fundamental roles in plant development (Axtell and Bowman, 2008; Cho et al., 2012; Cuperus et al., 2011; Fahlgren et al., 2010; Taylor et al., 2014). It is also one of the most abundant miRNAs in *Arabidopsis* (Breakfield et al., 2012; Kozomara and Griffith-Jones, 2011), where at least 10 loci encode *MIR156* family members (including *MIR156i* and *MIR156j*) (Breakfield et al., 2012; Kozomara and Griffith-Jones, 2011; Reinhart, 2002). miR156 displays a time-dependent expression profile which reaches its maximum at the seedling stage, followed by a gradual decrease (Wu et al., 2009). However, the mechanism by which this pattern is accomplished remains unclear (Wu et al., 2009; Yang et al., 2013). Transcript levels of genes targeted by miR156 also have a gradual trend, which anticorrelates to that of the microRNA by increasing with the age of a plant (Cardon et al., 1999; Wang et al., 2009; Wu and Poethig, 2006; Wu et al., 2009; Yamaguchi and Abe, 2012).

Targets of miR156 have been identified as important regulators of developmental phase change. Transcripts of numerous SQUAMOSA PROMOTER BINDING PROTEIN LIKE (SPL) transcription factors contain miRNA156 recognition sites (Birkenbihl et al., 2005; Chen et al., 2010; Gandikota et al., 2007; Guo et al., 2008; Schwab et al., 2005; Shikata et al., 2009; Wu and Poethig, 2006). SPLs are evolutionary conserved and restricted to the plant kingdom (Cardon et al., 1999; Guo et al., 2008; Xing et al., 2011) and play roles in major developmental processes, including seed germination, juvenile-to-adult and floral phase transitions (Gandikota et al., 2007; Shikata et al., 2009), leaf and plastochron development

(Wang et al., 2009; Wang et al., 2008), trichome formation and distribution (Wu and Poethig, 2006; Wu et al., 2009), fruit ripening (Manning et al., 2006), as well as copper homeostasis (Yamasaki et al., 2009). Even though they are closely related and partially redundant, SPLs also have specific functions (Cardon et al., 1999; Kim et al., 2012; Schwarz et al., 2008; Shikata et al., 2009; Wu et al., 2009; Yamaguchi and Abe, 2012).

Constitutive overexpression of *MIR156* has been reported to lead to a moderate delay in flowering, accompanied by production of an increased number of juvenile leaves (Schwab et al., 2005; Wu and Poethig, 2006). Conversely, plants overexpressing miR156 mimicry targets (*35S:MIM156*), which results in sequestration of miR156, display accelerated juvenile-to-adult transition and early flowering (Franco-Zorrilla et al., 2007; Todesco et al., 2010), similarly to effects caused by constitutive overexpression of resistant forms of miR156-targeted SPLs (Kim et al., 2012; Wu and Poethig, 2006).

It has been suggested that synchronized action of multiple miR156-targeted *SPL* genes and *SPL8* (not containing a miR156 target site) is required for proper developmental timing and full fertility of *Arabidopsis* (Xing et al., 2013; Zhang et al., 2007).

While the events taking place downstream of *MIR156* transcription have been described, the mechanisms directly regulating transcription of miR156 precursors have not been studied in detail yet (Huijser and Schmid, 2011; Yamaguchi and Abe, 2012). It has been suggested that leaves can act as source of a signal which represses *MIR156* transcription (Yang et al., 2011). In addition, level of miR156 was also reported to be influenced by temperature, CO_2 (May et al., 2013) and sugar (Yang et al., 2013). More recently, a function for the B3 domain transcription factor FUSCA3 in transcriptional regulation of miR156 via direct binding to the promoter region of *MIR156C* (Wang and Perry, 2013) was reported. Also, feedback by SPLs on regulation of miR156 precursor transcription was demonstrated (Fornara and Coupland, 2009; Wei et al., 2012). Such feedback connection might play an important role in the irreversible vegetative-to-reproductive phase transition (Wang et al., 2009; Yu et al., 2012).

SPLAYED (SYD) is one of the canonical SWI2/SNF2 chromatin remodeling ATPases of *Arabidopsis*. It was initially discovered in a genetic mutant screen for

phenotypic enhancers of plants defective in LEAFY (LFY) and has been demonstrated to play important roles in several aspects of plant development (Jerzmanowski, 2007; Wagner and Meyerowitz, 2002; Wu et al., 2012). SYD was reported to control transcription of the SAM maintenance regulator *WUSCHEL* (*WUS*) (Kwon et al., 2005) and to be required for promoting transcription of homeotic genes (Wu et al., 2012). Recent studies revealed that SYD can directly interact with LFY and SEPALLATA, acting antagonistically to Polycomb repression to regulate floral organ identity (Wu et al., 2012). Moreover, SYD was shown to be recruited to the promoters of several defense-related genes (Walley et al., 2008).

Numerous roles in developmental processes have been revealed for SYD. However, functions of SYD in regulating the dynamics of vegetative phase changes and its relation to the miR156 pathway have not yet been described. In this study we have investigated the connection between SYD and miR156 in the regulation of vegetative phase timing. We show that SYD acts upstream of miR156 by directly regulating transcription of its precursor mRNA and that it simultaneously interacts with the promoter regions of miR156 targeted and miR156 independent *SPL* genes (*SPL3* and *SPL8*, respectively). Thus, SYD directly modulates the balance between miR156-dependent and miR156-independent regulatory pathways during plant development.

RESULTS

In order to identify modulators of the epigenetically controlled *APUM9* locus in *Arabidopsis* (Yokthongwattana et al., 2010), we performed an EMS-based forward genetic mutant screen on an *APUM9* GFP reporter line (called silex, see Materials and Methods). In this screen we recovered two mutant alleles of *SPLAYED* (*SYD*): *syd-10*, carrying G to A single nucleotide polymorphism (SNP) at the last base of exon 22, which resulted in defective splicing of the *SYD* mRNA (Fig. S4.1A) and *syd-11*, containing a C to T SNP that caused an amino acid substitution (S1096F, Fig. S4.1B). Here, we report on the roles of SYD in the regulation of developmental timing.

We first compared the developmental phenotypes displayed by flowers of previously reported *SYD* mutant allele to the new allele we identified. Flowers of *syd-5* (SALK_023209) (Alonso et al., 2003; Bezhani et al., 2007) and *syd-10* mutant alleles exhibited pleiotropic phenotypes compared to wild type and the parental silex reporter line (Fig. S4.1C). Floral organ count analysis showed that the severity of phenotype was allele-dependent: *syd-10* generally displayed a weaker phenotype than *syd-5* (Fiume et al., 2010) (Table S4.1). Flowers of *syd-10*, as well as *syd-5* displayed variability in numbers of petals and stamens (from 1 to up 7). Also, flowers of both *syd* alleles generally had a decreased average number of stamens with the majority of flowers having less than 6, often 5 or only 4 of the same size (Table S4.1). Moreover both, *syd-5* and *syd-10* flowers often exhibited abnormalities in gynoecium development resulting in only one carpel, or completely missing carpels with only the septum being present (Table S4.1). 31% of *syd-5* flowers and only 3% of *syd-10* flowers had misshapen open carpels with visible ovules. Development of uneven carpels, or presence of only one of them correlated with severe curving of the gynoecium. These results indicate that *syd-10* is a weaker *SYD* mutant allele than *syd-5* (Fig. S4.1C, Table S4.1).

We observed that under long day conditions *syd-10* plants displayed a moderate delay in flowering initiation, compared to wild type (Fig. S4.1D). Rosette leaves of *syd-10* exhibited juvenile characteristics, such as round shape and lack of serrations.

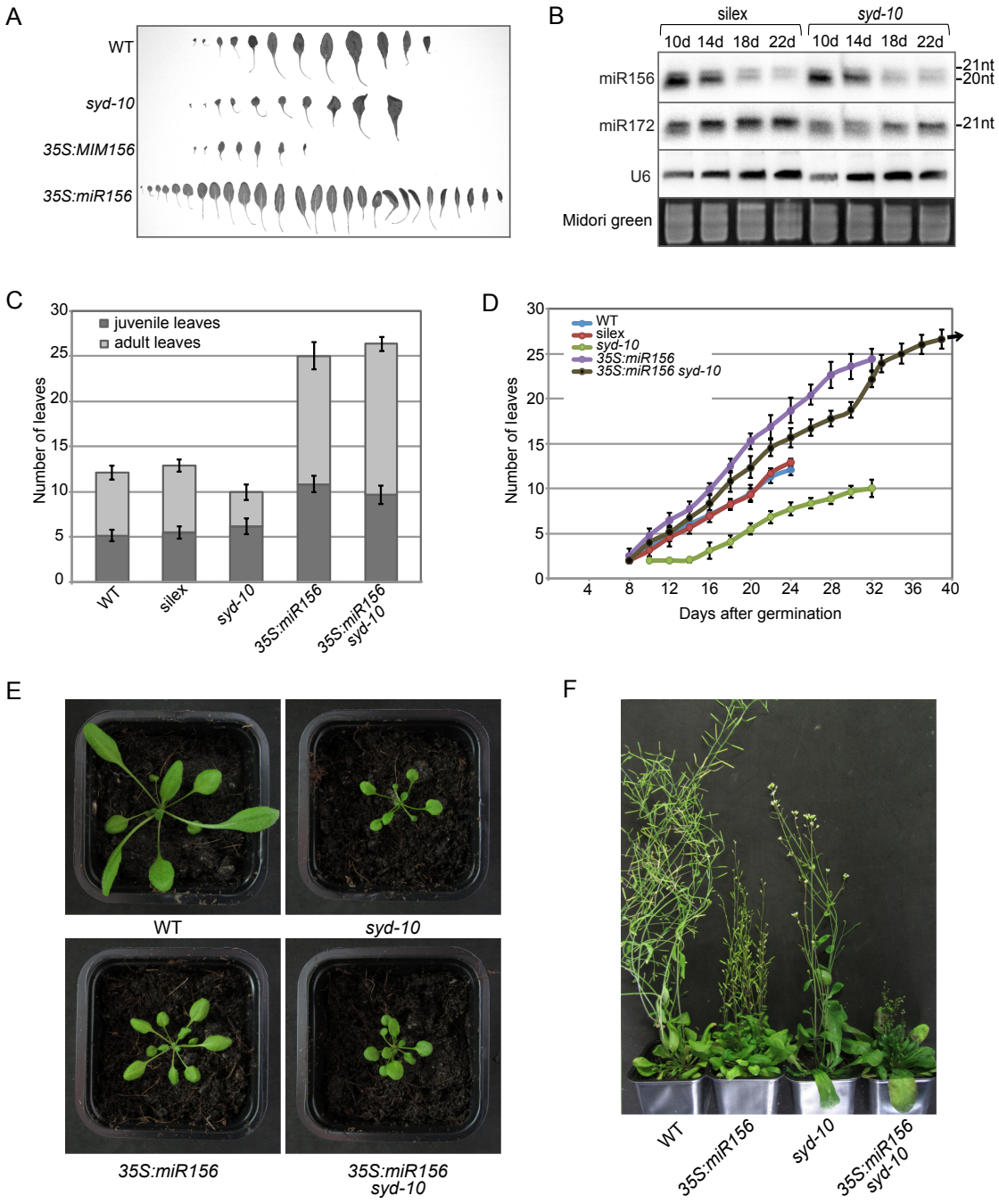


Figure 4.1 Vegetative phase change and flowering time is affected in *syd-10*

(A) Shape of rosette leaves of wild type (WT), *syd-10*, *35S:MIM156* and *35S:miR156* plants. (B) Northern blot detecting miR156 and miR172 on RNA extracted from silex and *syd-10* seedlings 10, 14, 18 and 22 DAG. Detection of U6 and the Midori Green stained gel serve as loading controls. (C) Quantification of the juvenile-to-adult phase transitions of wild type (WT), parental line (silex), *syd-10, 35S:miR156* and *35S:miR156 syd-10* plants. (D) Total rosette leaf number and flowering time (corresponding to the last point of each curve) for WT, silex, *syd-10*, *35S:miR156* and *35S:miR156 syd-10*. Error bars represent standard deviation (s.d.), where n=20; the experiment was repeated 3 times, the arrow indicates that 20% of *35S:miR156 syd-10* plants did not complete their life cycle even after 60 days. The statistical significance

of results was estimated by the Student's t test, where $p \leq 0.05$. (E) Rosettes of 21 days old plants grown under long day conditions. (F) Adult flowering plants of WT, *35S:miR156, syd-10*, and *35S:miR156 syd-10* (50 DAG).

Also, *syd-10* developed fewer rosette leaves (Fig. 4.1A). In *Arabidopsis*, leaf initiation rate and vegetative transition have been reported to lie under the control of miR156 (Wang et al., 2008; Wu and Poethig, 2006). Coherently with previously published data, we observed that plants overexpressing miR156 (*35S:miR156*) had an increased number of rosette leaves (Wu and Poethig, 2006), whereas plants with a decreased level of miR156 (*35S:MIM156*) initiated fewer leaves (Franco-Zorrilla et al., 2007; Wang et al., 2009) (Fig. 4.1A).

Because we observed changes in leaf number and developmental timing in *syd-10*, we hypothesized that this may be the result of changes in the dynamics of miR156 accumulation and/or of its target genes. To test this we performed northern blots in order to detect small RNAs from plants at the age of 10, 14, 18, and 22 days after germination (DAG, Fig. 4.1B). We found that the level of miR156 in the parental silex line and *syd-10* decreased at a similar rate. However, in *syd-10* miR172 started at a lower level and did not accumulate to such a high level as in the parental line 18 DAG. This indicated a possible missregulation of the complex miR156 – miR172 pathway in *syd-10* (Fig. 4.1B).

To compare the effects of miR156 and SYD on vegetative phase change and leaf initiation, we investigated the dynamics of juvenile-to-adult phase change and leaf numbers in wild-type plants, *syd-10* and the *35S:miR156* overexpressor line (Wu and Poethig, 2006). Compared to controls, *syd-10* had a significantly slower leaf initiation rate and it also had a lower total rosette leaf number at flowering, whereas we observed the opposite for *35S:miR156* (Fig. 4.1C,D, Fig. S4.2A.B). However, despite the delay in development with respect to the calendar age, *syd-10* initiated the same number of juvenile leaves as wild type (Fig. 4.1C). In contrast, *35S:miR156* plants were affected in both, total leaf number and number of juvenile leaves (Fig. 4.1C). Introgression of *syd-10* into *35S:miR156* led to an increase of total and juvenile leaf numbers, in a manner similar to wild type (Fig. 4.1C-E).

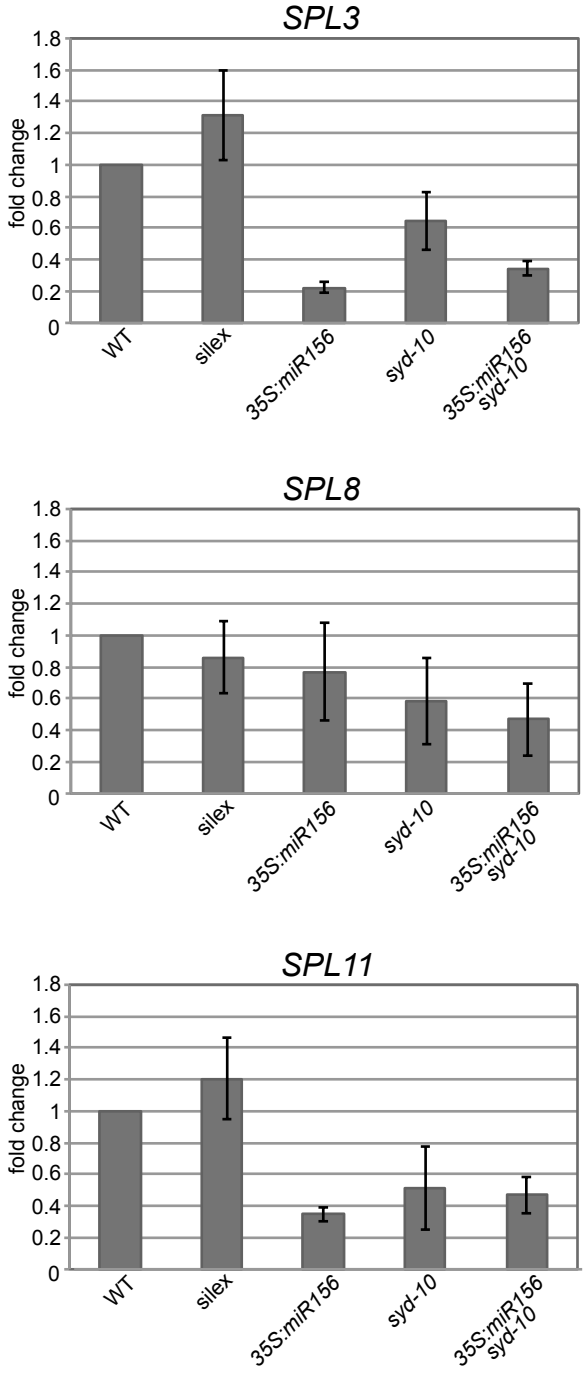


Figure 4.2 Expression of *SPL* genes in seedlings

qRT-PCR quantification of *SPL3, SPL8* and *SPL11* mRNA levels in seedlings of *syd-10, 35S:miR156* and *35S:miR156 syd-10* compared to the parental silex line. *UBQ10* was used as reference gene. Error bars show s.d. of three biological repeats.

At the same time, flowering of both, *syd-10* and *35S:miR156* was delayed compared to wild type and parental silex line (8 to 10 days delay). Introgression of *syd-10* into *35S:miR156* resulted in plants with an enhanced delay in flowering time: on average 39 days, compared to 32 days for *35S:miR156* and *syd-10* alone whereas it was 24 days for wild type plants and plants of the parental silex line (Fig. 4.1D,F, Fig. S2B). Notably, at least 20% of the *35S:miR156 syd-10* double mutant plants initiated flowering only after 65 days under long day conditions. Therefore, we observed an enhanced delay in flowering initiation time in *35S:miR156 syd-10* (Fig. 4.1F). In all of these experiments the *syd-10* and *syd-5* alleles behaved similarly (Fig. S4.2A,B).

To check whether mRNA levels of miR156-targeted *SPL* genes were changed in *syd-10*, we performed real-time PCR experiments on RNA extracted at 18 DAG. mRNA levels of the miR156 targeted *SPL3* and *SPL11* transcripts were reduced in *syd-10* as well as in *35S:miR156* plants or *35S:miR156 syd-10* (Fig. 4.2, Fig. S4.3A). At the same time, *SPL8* that is not targeted by miR156 was not significantly affected in *35S:miR156* and *syd-10* but we observed some down regulation in *35S:miR156 syd-10* (Fig. 4.2).

However, due to affected timing of vegetative-to-reproductive phase change of *syd*-10, it was difficult to set up a proper comparison of plants at an equivalent developmental stage. Since miR156 and *SPL* genes also play roles during the reproductive stage (Gandikota et al., 2007; Nag and Jack, 2010; Xing et al., 2013), we decided to focus our next analyses on inflorescence tissues, which are developmentally more determined. Therefore, to estimate the abundance of miR156 in tissues at the same developmental stage, we tested levels of miR156 and its target genes in inflorescences of *syd-10* and control lines. The *ago1* line was used as control for reduced abundance of the miRNA (Morel et al., 2002; Smith et al., 2009). Northern blot quantification of miR156 in inflorescence tissues showed that it was significantly decreased in both *syd-5* and *syd-10* alleles (Fig. 4.3A,B). We also observed lower miR156 levels in upper parts of *syd-10* stems (Fig. S4.4A,B). At the same time, miR172 was not significantly affected in this tissue (Fig. 4.3A, Fig. S4.4A).

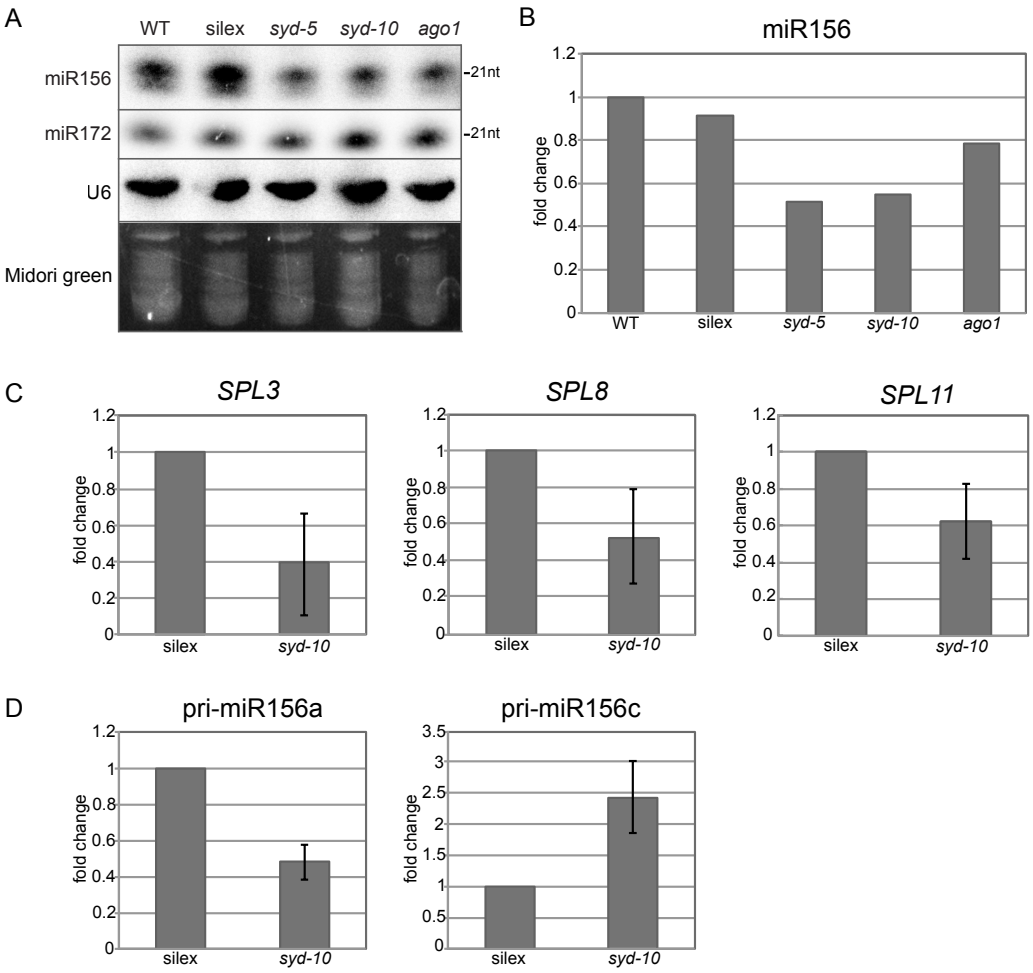


Figure 4.3 Accumulation of miR156 and transcript levels of its target genes in inflorescences of *syd* mutant alleles

(A) Northern blot of miR156 and miR172 in inflorescences of *syd-5* and *syd-10,* compared to wild type (WT) and parental line (silex), *ago1* was chosen as a control for reduced miR156 accumulation (Smith et al., 2009); U6 was used as a loading control. (B) U6 normalized quantification of miR156 levels shown in (A). (C) Fold change in mRNA levels of *SPL3, 8, 11* in inflorescences of *syd-10* compared to parental line; (D) Fold change in expression of miR156 precursors isoforms in inflorescences of *syd-10* compared to silex parental line, error bars show s.d. of three biological repeats.

To investigate possible effects of changes in the microRNA pathway and their connection to the phenotype of flowers in *SYD* mutant plants, we compared mRNA levels of selected *SPL* genes in inflorescences of the parental silex line and *syd-10.* Indeed mRNA levels of *SPL3* and *SPL11* were changed in *syd-10*, but contrary to the

expected elevation (as due to a decrease in miR156 accumulation), both *SPL3* and *SPL11* were down regulated in *syd-10* and *syd-5* (Fig. 4.3C, Fig. S4.3B). In addition, expression of *SPL8*, which is not targeted by miR156, was also decreased in inflorescences of *syd-10* and *syd-5* (Fig. 4.3C, Fig. S4.3B for *syd-5*).

Furthermore, to study upstream events of miR156 biosynthesis, we tested whether transcription of miR156 precursors was affected in *syd-10* inflorescences. Expression of precursors pri-miR156a and pri-miR156c was changed in *syd-10*. Surprisingly, expression of pri-mir156a was decreased whereas the level of pri-miR156c was elevated (Fig. 4.3D, see Figure S4.3C for *syd-5*).

Taking into account the indications that the miR156 pathway may be miss-regulated in *syd-10*, we studied the possibility of SYD directly controlling transcription of *MIR156* genes. First, we examined the expression patterns of *SYD* and miR156. For this, a *SYD:GUS* reporter line which included 2.4kb of the *SYD* promoter region was generated. In agreement with previous reports (Bezhani et al., 2007; Su et al., 2006), *SYD* was highly expressed in young leaves, vasculature of mature leaves and inflorescence, including upper parts of inflorescence stems (Fig. S4.5A). Results of *in situ* hybridization on inflorescence tissues indicated that the *SYD* mRNA accumulation pattern overlaps with that of miR156 (Fig. S4.5B).

Finally we tested whether SYD could directly interact with the promoter regions of *MIR156* and missregulated *SPL* genes. In order to assess this, ChIP-qPCR using a SYD-specific antibody was performed on inflorescence tissues (Fig. 4.4). Inflorescences of *syd-10* were used as a negative control. Occupancy of SYD in promoter regions of target genes was estimated as fold enrichment over a pull down with an unspecific antibody (IgG) and compared to enrichment at *EIF4A* a locus previously reported not to be bound by SYD (Kwon et al., 2005). The promoters of *MIR156A* and *MIR156C* showed significant enrichment of SYD. Furthermore we observed that both, the promoters of the miR156 targeted *SPL3* and the miR156 independent gene *SPL8* were also bound by SYD (Fig. 4.4).

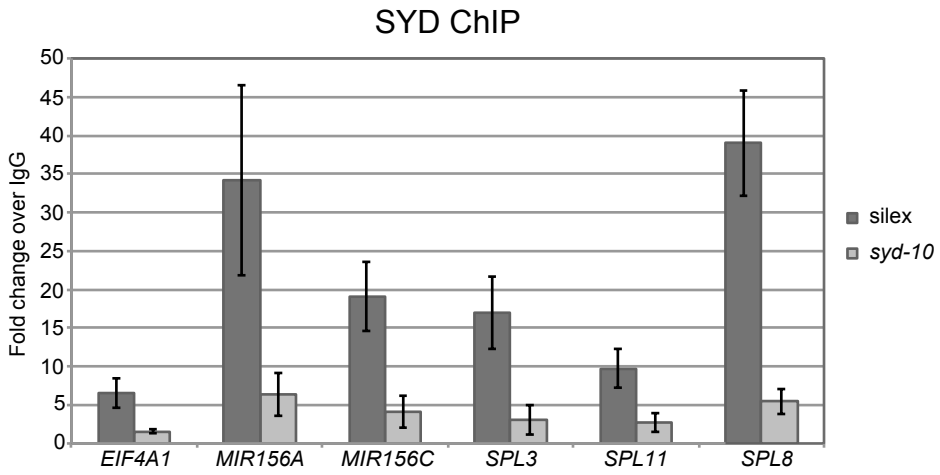


Figure 4.4 SYD physically interacts with promoter regions of *MIR156* and *SPL* genes

ChIP analysis of SYD binding to different promoters quantified by qPCR in silex and *syd-10* inflorescences. The bars indicate fold enrichment over that obtained by performing ChIP using an unspecific antibody (IgG). Error bars show s.d. of three biological replicates.

DISCUSSION

The *syd* mutant was first described as an enhancer of the *lfy* flower development phenotype (Wagner and Meyerowitz, 2002). As a reflection of its extensive functions in development, plants deficient in SYD display pleiotropic developmental phenotypes and severely reduced fertility. Comparison of the flower phenotypes of the two *syd* mutant alleles used in this study showed that flowers of both *syd-5* and *syd-10* were affected (Fig. S4.1C). Severity of the flower phenotypes was allele-dependent, showing that *syd-10* was a weaker allele than *syd-5*, which was consistent with our finding that *syd-10* still accumulates detectable albeit strongly reduced amounts of SYD protein (Fig. 3.9). The developmental phenotypes we observed in flowers included changes in sepal and petal numbers and defective gynoecium patterning. These defects can be attributed to the function of SYD in the direct regulation of flower patterning genes (Kwon et al., 2005; Wagner and Meyerowitz, 2002; Wu et al., 2012). On the other hand, proper patterning of gynoecium has also been previously reported to be regulated by SPL8 and miR156-targeted SPLs in a redundant manner (Gandikota et al., 2007; Xing et al., 2013; Xing et al., 2010). Even though no direct link between SYD and the miR156 regulatory pathway has been demonstrated before, evidences supporting this connection have recently emerged. Among them are the direct regulation of *LFY* expression by SPL3 (Yamaguchi et al., 2009), and occupancy of LFY in the proximity of microRNA genes (Moyroud et al., 2011; Winter et al., 2011). Furthermore roles for both miR156 and SYD in the control of flower induction have been reported (Kim et al., 2012; Wagner and Meyerowitz, 2002; Wu and Poethig, 2006).

We did not detect changes in miR156 levels in *syd-10* leaves when we compared them to the parental silex line of the same calendar age (Figure 4.1B). However changes in leaf shape and leaf number of *syd* plants suggested that the miR156 pathway might be disturbed (Fig.4.1C-F). Also, similarly to *35S:miR156* transgenic lines, flowering of *syd-5* and *syd-10* was delayed under long day conditions (Fig. 4.1D,F, Figs. S4.1D, S4.2B). In contrast to the miR156 overexpressor line, where numbers of both juvenile and adult leaves were increased, *syd* plants had a decreased total number of leaves. This decrease was associated with a reduced number of adult leaves and thus, a shortened adult stage. Notably, we observed no change in the number of juvenile leaves in *syd-10* (Fig. 4.1C, Fig. S4.2A). Similar

phenotypic effects have previously been reported for the *35S:LFY* overexpressor line (Telfer and Poethig, 1998). However, due to the general delay of development in *syd-10*, the biologically shortened adult stage (from formation of first adult leaf until initiation of flowering) requires the same calendar time as plants of the control line, indicating that SYD is important for proper timing of the adult stage.

In order to study possible effects of increased miR156 levels on the *syd-10* phenotype, we introgressed *syd-10* into the *35S:miR156* line. We observed that *35S:miR156 syd-10* displayed an additive delay in initiation of flowering compared to *syd-10* and *35S:miR156* alone (Fig. 4.1D,F). This suggests that miR156 and SYD act in separate pathways to control flowering time, which is supported by previous reports showing that SYD can influence expression of *FLOWERING LOCUS T* (*FT*) (Su et al., 2006). However introgression of *35S:miR156* into *syd-10* rescued both, leaf initiation rate and leaf number, suggesting that overproduction of miR156 in *35S:miR156 syd-10* had a dominant effect on leaf initiation. Taken together these results suggest that SYD may take part in the regulation of the miR156 pathway but that it is also involved in the regulation of developmental phase change independently of miR156.

To further investigate possible connections between the miR156 and the SYD pathways, we compared the mRNA levels of miR156 targeted *SPL* genes. Surprisingly, we observed reduced *SPL* mRNA levels without concomitant changes in miR156 accumulation (Fig. 4.2, Fig. S4.3A). This suggested that some miR156-targeted *SPL* genes also lie under the control of another, miR156-independent mechanism influenced by SYD. mRNA levels of the miR156-independent *SPL8* gene (Xing et al., 2010) was reduced in *35S:miR156 syd10* plants, further indicating that SYD can act independently of miR156 (Fig. 4.2).

The developmental delay displayed by *syd-10* plants is important to take into account when mRNA and miRNA levels are compared. Therefore, to be able to assess a developmentally well-defined tissue we further pursued our study in inflorescences. We observed that, compared to control lines, miR156 levels were significantly decreased in inflorescences of *syd-5, syd-10* and *ago1* (Fig. 4.3A, B). Since we detected a decrease in miR156 accumulation we expected increased mRNA levels for the miR156 targeted *SPL* genes. Surprisingly, the set of miR156-controlled *SPL* genes we tested was also down regulated in *syd-10* inflorescence (Fig. 4.3C, Fig. S4.3B). To exclude deficient pri-miR156 processing we assessed the levels of miR156

precursors. We detected decreased levels for pri-miR156a and pri-miR156h and elevated levels of pri-miR156c (Fig. 4.3D, Fig. S4.3C). Elevation of miR156c precursor expression did not seem to correlate with the general decrease in the amount of miR156 observed on northern blots. However this may be explained by the fact that pri-miR156a is the predominant source for miR156 (Yang et al., 2013) (Fig. S4.3D). This suggests that reduced *MIR156A* transcription lead in an overall reduction in miR156 accumulation. Therefore, local elevation of pri-miR156c would not bring a detectable impact on the background of pri-miR156a down regulation. On the other hand, the opposite changes in expression of these miR156 precursors correlate with the complex *syd-10* phenotype, combining characteristics of both, overexpression and down-regulation of this microRNA: juvenile shape of rosette leaves in combination with the decreased leaf number, respectively (Franco-Zorrilla et al., 2007; Huijser and Schmid, 2011; Wang et al., 2009). A further explanation for this effect could be the presence of a feedback loop coming from downstream genes. The existence of such a loop was reported for SPL15 and miR156b (Wei et al., 2012). Therefore it may well be that expression of some of miR156 isoforms, including miR156c can be regulated by transcription factors of the SPL group. At the same time, down-regulation of tested miR156-targeted *SPL* and miR156-independent genes in *syd-10* indicated that SYD could directly regulate expression of some of these genes.

Considering the overlap in tissue-specific expression patterns of *SYD* and miR156 (Fig. S4.5B), we tested the possibility of a direct interaction of SYD with promoter regions of selected *MIR156* and *SPL* genes. Indeed we observed a high level of enrichment of SYD at tested promoter regions of several *MIR156* and *SPL* genes. This result shows that SYD can directly modulate transcription of the *MIR156A* and *MIR156C*. Interestingly, SYD enrichment was higher in the promoter region of *MIR156A* than *MIR156C*. At the same time, FUSCA3 (FUS3) has recently been reported to take part in transcriptional regulation of *MIR156* expression by preferentially occupying the *MIR156C* gene (Wang and Perry, 2013). On the other hand we detected significant enrichment for SYD in the promoter regions of *SPL3* and *SPL8* (but not *SPL11*). Notably, SPL3 has been reported to act as a direct upstream activator of *LEAFY* and *APETALA*, two important floral meristem identity regulators (Mandel and Yanofsky, 1995; Ripoll et al., 2011; Weigel et al., 1992) and interaction partners of SYD (Smaczniak et al., 2012; Wu et al., 2012), thus linking the expression of these factors. Supporting our observations it has been reported that

LFY was found to be associated to *MIR156* and *SPL* genes which could promote recruitment of SYD at these loci (Moyroud et al., 2011; Winter et al., 2011).

However, although several genes subjected to regulation by SYD have been identified (Kwon et al., 2005; Kwon et al., 2006; Wu et al., 2012), features that define SYD target recognition still remain elusive. Unlike its close homolog BRAHMA (BRM), SYD does not contain a bromodomain, which is required for binding acetylated histones (Jerzmanowski, 2007). Therefore recognition of target loci by SYD may be dependent on other factors. In line with this suggestion, LFY has been reported to play a role in recruitment SYD to *AP3* and *AG* regions (Wu et al., 2012). At these loci SYD can trigger the ejection or sliding of nucleosomes thereby leading to removal of repressive H3K27me3 histone marks. This may permit recruitment of proteins of the Trithorax group to the region thus facilitating further transcription of a target gene.

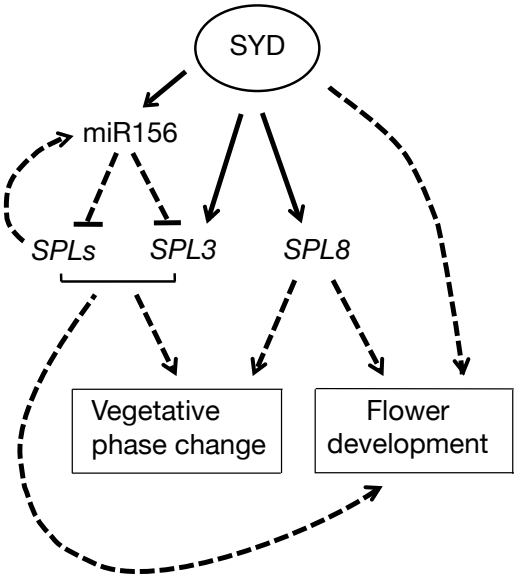


Figure 5. Model of SYD influencing the vegetative phase change

SYD contributes to the control of the miR156-dependent developmental phase change by directly influencing transcription of miR156 precursors and the miR156 target *SPL3*. In addition, SYD interacts with the promoter region of *SPL8*, which is not targeted by miR156. Therefore, SYD regulates the balance between miR156-dependent and miR156-independent developmental phase change pathways in *Arabidopsis*. Dashed lines indicate previously reported connections whereas full lines mark the interactions demonstrated by this study.

In summary, we establish SYD as an important transcriptional modulator of the *MIR156A* and *MIR156C* genes, the miR156-targeted *SPL3* gene and the miR156-independent *SPL8* gene. Moreover, we showed that SYD operates by directly binding to the respective promoters. Our data reveals a new function for SYD as regulator of the dynamic balance of expression of miR156 dependent and miR156 independent genes and thereby proper timing of development and vegetative phase change in *Arabidopsis* (Fig. 4.5). We propose that SYD may act as a direct regulator of temporal synchronization of miR156-dependent and miR156 independent regulatory pathways. Also, the high conservation of miR156 (Cho et al., 2012; Meyers et al., 2008; Taylor et al., 2014), miR156-target genes (Birkenbihl et al., 2005; Cardon et al., 1999), and SWI2/SNF2 ATPases (Jerzmanowski, 2007; Knizewski et al., 2008) in the plant kingdom suggests that this mechanism may also be present and act in regulation of developmental timing in other plant species.

MATERIALS AND METHODS

Plant material

All plants used in this study are from the Columbia accession. The silex GFP reporter line was obtained from a collection created by The Institute for Genomic Research (J. Craig Venter Institute, line AGRAC-60-1-1) (Xiao et al., 2010). Mutants used in this work were: *syd-5* (SALK_023209) (Alonso et al., 2003; Bezhani et al., 2007), *syd-10* (this study), *35S:miR156* (Wu and Poethig, 2006), *35S:MIM156* (Franco-Zorrilla et al., 2007) and *ago1-27* (Morel et al., 2002). Plants were grown in Sanyo MLR-350 chambers at 24°C with 16 hours light. Plants for *in situ* hybridization were grown at long day conditions (16h light), at 21°C.

Leaf counting and floral organ counting

Number of rosette leaves on plants during the period from germination till flowering was counted every 48h. The first leaf with abaxial trichomes was marked in every genotype tested as described in (Telfer et al., 1997). The experiment was repeated 3 times, each pool including 15 to 20 plants (10 to 15 for *35S:miR156 syd-10* plants). Floral organ counting was performed as described in (Fiume et al., 2010).

Quantitative RT-PCR

Total RNA from 100mg of fresh leaf tissue was isolated using the innuPREP Plant RNA Kit (Analytik Jena) following the manufacturers protocol. 500 ng of RNA were used for cDNA synthesis using the iScript cDNA synthesis kit (Bio-Rad). mRNA levels were measured by quantitative PCR (qPCR) on a Light-Cycler 480 (Roche), using the SYBR Green I Master Mix. Primer sequences are presented in Supplementary Table A. Melt curve analysis confirmed that no primer dimers were formed during amplification. The absence of genomic DNA was confirmed by performing qPCRs on non-reverse transcribed RNA. Expression results were normalized using *UBQ10* as a reference gene. All qPCRs were performed on three biological repeats.

RNA preparation for northern blots

Total RNA was isolated from 250 – 300 mg of fresh plant material with the mirVana miRNA Isolation Kit (Ambion). For small RNA blots, 8 - 10 µg of the sRNA fraction with equal volume of loading buffer (95% formamide, 20mM EDTA pH 8, 0.05% bromophenol blue, 0.05% xylene cyanol: for 10 ml, 9.5 ml formamide, 0.5 ml 200mM EDTA pH 8) were denatured at 90° for 2 min and loaded on 15% polyacrylamide gel with 0.5g/ml urea. The RNA was transferred to a Hybond N+ membranes in TBE 1x buffer, 10V at 6°C overnight. Membranes were UV crosslinked with a dose of 1400 joules.

GUS reporter line and staining

The Promoter region of *SYD* (*AT2G28290*) was PCR amplified (see Supplementary Table S2 for primer sequences) and cloned into the pCAMBIA1304 expression vector (GenBank:AF234300.1). *Arabidopsis* plants were transformed by floral dipping (Clough and Bent, 1998). Staining for GUS was performed by vacuum infiltration of sampled plant material with the Staining solution (50mM NaPi, pH7.0, 0.5mM $K_3Fe(CN)_6$, 0.5mM $K_4Fe(CN)_6$, 10mM EDTA, 1mM x-Gluc) followed by incubation at 37°C overnight. Tissues were distained by multiple washes with 70% ethanol and stored in 50% glycerol (Jefferson et al., 1987).

***In situ* hybridization**

Accumulation of miR156 and *SYD* mRNAs in inflorescences were studied by non-radioactive *in situ* hybridization (Carles et al., 2010). An LNA probe for miR156 (G*TGC*TCA*CTC*TCT*TCT*GTCA/3Dig_N, where locked nucleotides are marked with an asterisk) was obtained from EXIQON (http://www.exiqon.com/ls). An antisense probe for *SYD* mRNA was designed in the N-terminal region of the gene, as described in (Wagner and Meyerowitz, 2002).

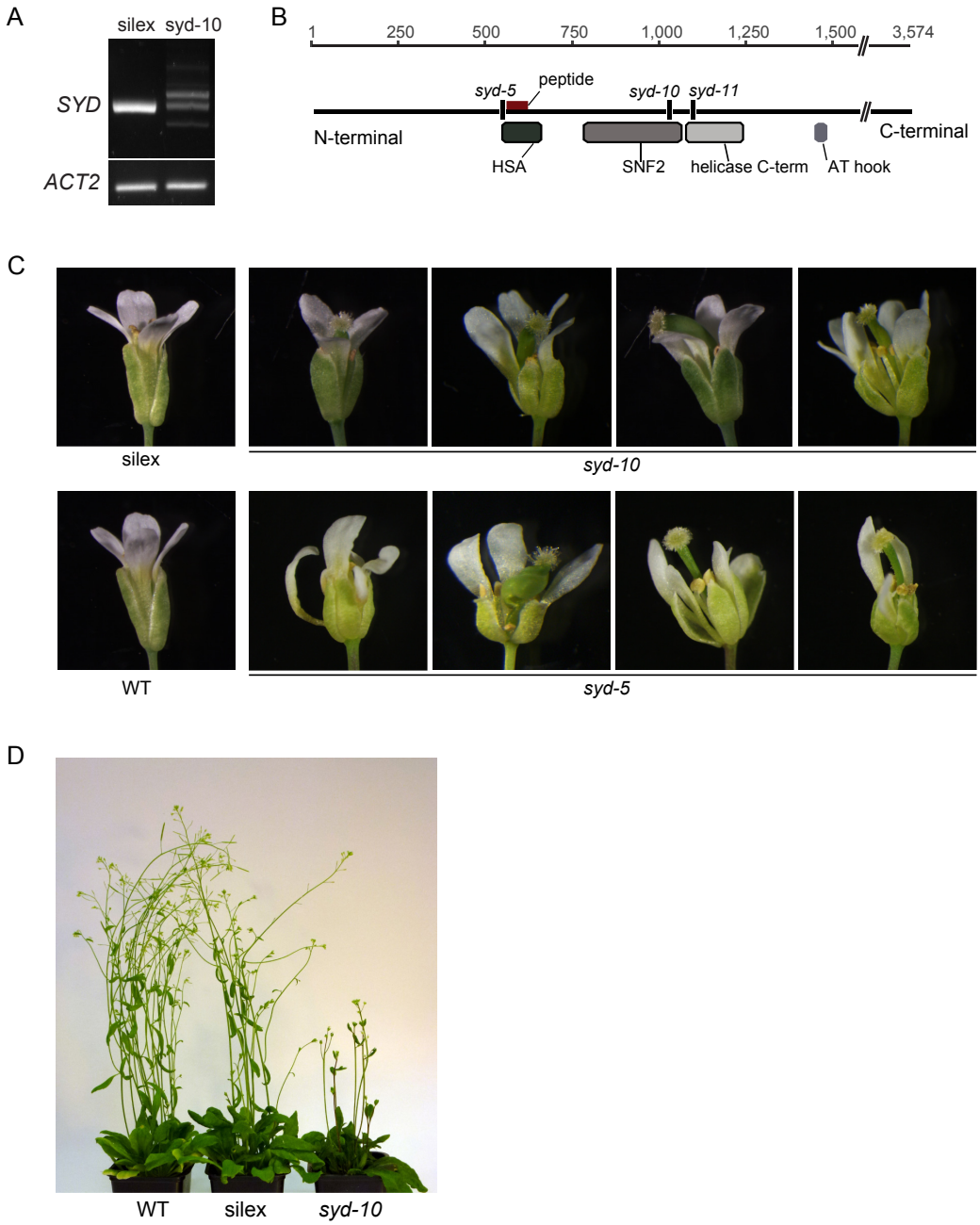
Chromatin immunoprecipitation

SYD-specific antiserum was raised in rabbits using a SYD-specific peptide (MKEERQRRIRERQKE-C, EZBiolab, Supplementary Figure S5A). Specificity of the antiserum was confirmed by Western blots on sonicated nuclei fractions. Nuclei

were extracted from 0.5g of fresh *Arabidopsis* tissue (inflorescences) as described in (Jaskiewicz et al., 2011), using protease inhibitor (Halt Protease Inhibitor Cocktail, EDTA-Free (100X), Product No 78437) and 20mM PMSF. Pellets were sonicated with Bioruptor UCD-200 to fragments of 0.3 – 0.5kb. 40µl of denatured samples with equal volume of 2x SDS loading buffer was run on a 7% polyacrylamide gel. Further, membranes were probed using SYD antiserum (1:500) or H3 antibody (pAB-060-050, Diagenode, as a loading control), followed by probing with secondary anti-rabbit HRP (1:30 000). The detection was performed with CDP-star reagent (NEB #N7001). For ChIP, plant material was cross-linked in infiltration buffer (13,69 g Sucrose, 1ml PMSF 100 mM, 1ml Tris/HCl 1M pH8, 200µl EDTA 0.5M, 2.7 ml formaldehyde 37% per 100ml) in the proportion of 40 ml of buffer per 0.5g of material. Infiltration was performed on ice (3 times, 10 min), 2ml of 2M Glycine was added to stop the fixation and infiltrated for 5 minutes. Extraction of nuclei and sonication were performed as described above (Jaskiewicz et al., 2011) with protease inhibitor (Halt Protease Inhibitor Cocktail, EDTA-Free (100X), Product No 78437) and 20mM PMSF. 10mg of Chromatin was used for immunoprecipitation with specific antibody on Protein A agarose beads (Roche, product No 11134515001) and protease inhibitor (Halt Protease Inhibitor Cocktail, EDTA-Free (100X), Product No 78437) overnight at 4°C. The bound fraction was washed 3 times with a Washing buffer (25mM Tris/HCl, pH 8.0, 5mM EDTA pH 8.0, protease inhibitor 1x), 3 times with LiCl buffer (250 mM LiCl, 10 mMTris-HCl pH8.0, 1 mM EDTA pH8.0, 0.5% NP-40 (Igepal), 0.5% Na deoxycholate, protease inhibitor 1x), and once with a TE buffer. All washing steps were performed at 4°C. Elution of precipitate was performed by adding 100 µl of Glycine elution buffer (0.1M glycine, 0.5M NaCl, 0.05% Tween-20, pH2.8) to the beads pellet. De-crosslinking was performed by adding 2ul of RNAase A 10mg/ml (1h incubation at 37°C) and 3µl of Proteinase K, 1mg/ml (3h, 65°C). Precipitated DNA was extracted with High Pure PCR Cleanup Micro Kit (Roche, Product No 04983912001).

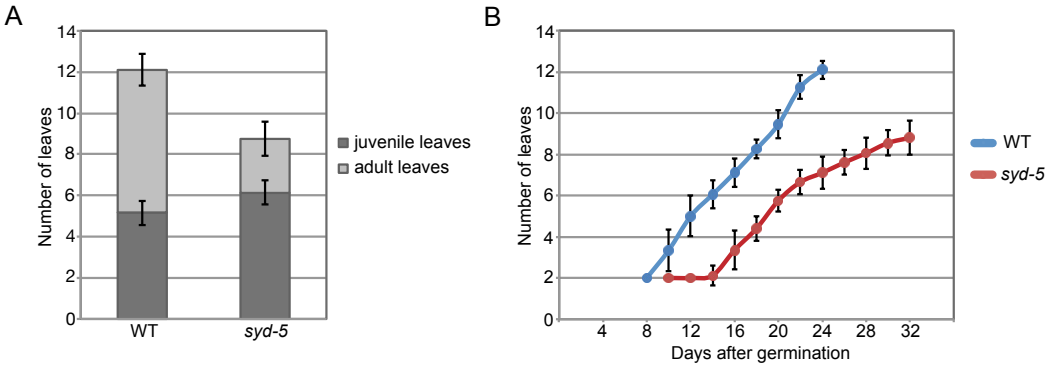
Standard curves for qPCR were built based on amplifications of dilution series from pooled input samples. Binding of SYD protein to a region was estimated by fold difference in percentage of enrichment over input by the specific antibody over IgG and compared to that in the proximal region of *EIF4A* (negative control) (Kwon et al., 2005). Error bars represent a standard deviation of 3 biological repeats.

SUPPLEMENTARY INFORMATION



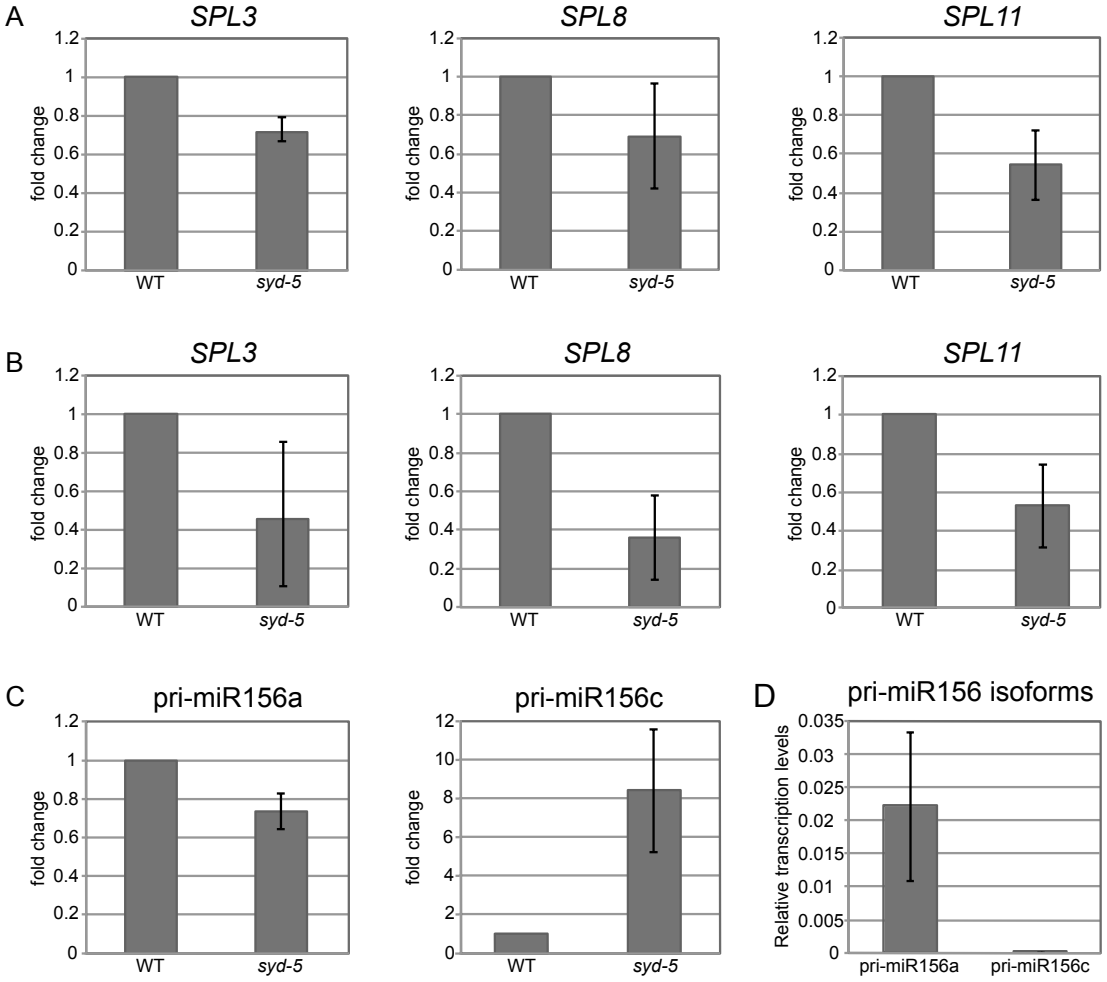
Supplementary Figure 1. Pleiotropic flower phenotype and flowering time delay displayed by the *syd* mutants

(A) Defective splicing of *SYD* mRNA in *syd-10* detected by RT-PCR with *SYD* specific primers (Supplementary Table S2). (B) Schematic map of the SYD protein. The different *syd* alleles are indicated: the T-DNA insertion site in *syd-5* and point mutations in *syd-10* and *syd-11*. The indicated peptide is the one that was used to raise anti-SYD antibody. (B) Phenotypes of flowers of the *syd-5* and *syd-10* alleles. (C) Adult plants of WT, silex and *syd-10* (40 DAG).



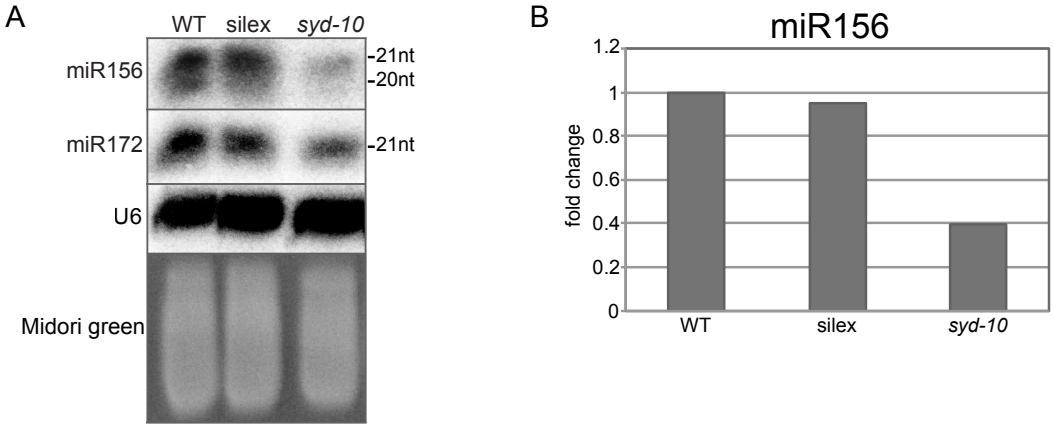
Supplementary Figure 2. Vegetative phase change and flowering time of *syd-5*

(A) Total rosette leaf number and flowering time (corresponding to the last point of each curve) of *syd-5,* and wild-type plants. (B) Juvenile-to-adult phase transition of *syd-5* and wild-type plants; error bars represent s.d., where n=20, the experiment was repeated 3 times. The statistical significance of results was estimated by the Student's t test, where $p \leq 0.05$.



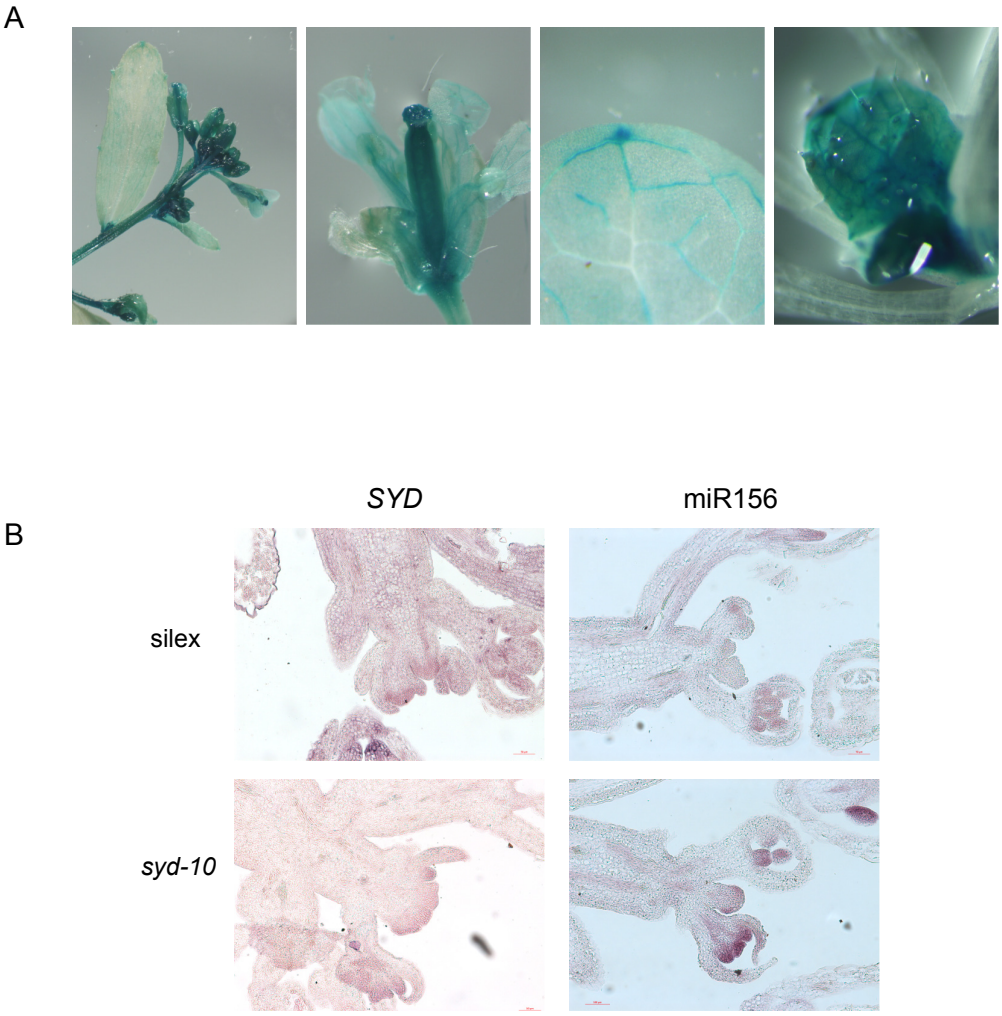
Supplementary Figure 3. Expression of miR156 precursors and its target genes in seedlings and inflorescences of *syd-5*

Fold change in mRNA levels of *SPL3, 8, 11* in seedlings (A) and inflorescences (B) of *syd-5* compared to wild-type. (C) Fold change in expression of miR156 precursor isoforms in inflorescences of *syd-5* compared to wild type (WT); (D) Relative abundances of selected miR156 precursor isoforms in inflorescences of wild-type plants; error bars show s.d. of three biological repeats.



Supplementary Figure 4. Reduced Accumulation of miR156 in stems of *syd-10*

(A) Northern blot of miR156 and miR172 in upper parts of stems of *syd-10* compared to wild type (WT) and silex; U6 was used as a loading control. (B) Fold change in accumulation of miR156 in upper parts of stems of *syd-10* compared to wild type (WT).



Supplementary Figure 5. Co-localization of miR156 and *SYD* mRNAs

(A) GUS stained inflorescence, single flower, adult and young leaves of *SYD:GUS* reporter plants. (B) *In situ* hybridization analysis using probes against miR156 and *SYD* detected in inflorescences of silex and *syd-10*.

Genotype	Sepals	Petals	Stamen	Carpels	Opened carpels (%)	Curving gynoecium (%)	Unequally sized petals (%)	Absent petals	N
Col	4,00	4,01±0,11*	5,98±0,21	2,00	not observed	not observed	not observed	not observed	90
syd-5	4,48±0,72	3,01±1,13	5,21±1,22	1,11±0,81	31%	44%	22%	8%	90
silex	4,01±0,11*	4,01±0,11*	5,97±0,23	2,00	not observed	not observed	not observed	not observed	90
syd-10	4,64±0,81	4,38±1,06*	5,2±0,78	0,96±0,81	3%	35%	15%	8%	90

Supplementary Table 1. Pleiotropic flower phenotype displayed by the *syd* mutants

Table, representing the average numbers of floral organs and morphological differences of *syd-5* and *syd-10* flowers. Represented error illustrates s.d. from the average, where n=90; * – detected in 1 flower.

GENERAL DISCUSSION

Several mechanisms that contribute to regulation of gene expression during the plant development have been described (Dunoyer et al., 2010; Martínez de Alba et al., 2013; Matzke and Mosher, 2014; Wu, 2013). Also, tight connections between the individual pathways have been recently demonstrated (Creasey et al., 2014; Numa et al., 2010; Nuthikattu et al., 2013). As proper patterning of the gene expression within the organs and tissues is important to insure their functions, the attention was brought to studying tissue-specific patterns of gene expression and mechanisms by which such specificity is defined (Farrona et al., 2011; Birnbaum et al., 2003; Brandt, 2005; Manavella et al., 2013; Schmid et al., 2005; Thain et al., 2002).

Recent reports indicated the important roles of epigenetic mechanisms in regulation of tissue-specific gene expression (Baubec et al., 2014; Kim et al., 2010). The initial aim of the designed project was to investigate how epigenetic factors may contribute to regulation of tissue specific gene expression. It was based on the results of previous studies, that demonstrated that expression of *APUM9* gene of *Arabidopsis* is controlled in a synergistic manner by Pol V a component of RdDM and MOM1 (Yokthongwattana et al., 2010). Recently, similar additive effect on expression of *APUM9* was reported for RdDM component DRD1 and chromatin remodeler DDM1 (Zemach et al., 2013). In order to unveil other epigenetic factors that are involved in this process, a transgenic GFP reporter line, that contains the endogenous *APUM9* promoter region (Xiao et al., 2010) was used.

This thesis includes the general characterization of the chosen GFP transgenic line. It also comprises the study on role of the chromatin remodeler SYD that was identified in the mutant screen in tissue specific silencing of the GFP transgene.

Mechanisms contributing to silencing of the GFP transgene in silex

Based on the previous report about the role of TGS in silencing of endogenous APUM9 (Yokthongwattana et al., 2010), it was initially assumed that in the silex line suppression of GFP expression may correlate with transcriptional silencing of the transgene. As loss of CHH methylation in *nrpe1* leads to release of GFP expression only in the lower part of inflorescence stems, it is likely that in addition to DNA methylation, other mechanisms contribute to silencing of the GFP

transgene in silex in other tissues. Our further results showed that mutations of components of the PTGS (*ago1, sgs3* and *dcl4*) also triggered the release of GFP expression (Baumberger and Baulcombe, 2005; Gasciolli et al., 2005; Morel et al., 2002; Mourrain et al., 2000; Qi et al., 2005). Therefore, both, TGS and PTGS play a role in regulation of expression of the transgene in silex. However, it is not clear, whether both systems act at the same time, or their impact is restricted to selected cell types or developmental phase of a plant.

Patterns of release of GFP expression in *ago1* and *dcl4* correlate with the predicted pattern of expression of these factors (eFP browser: http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi). It is likely that the ta-siRNA pathway and PTGS are acting to suppress GFP expression in these tissues. At the same time this suggests that both systems contribute to silencing of the transgene equally and in a complementary manner. This might mean that loss of function of a TGS factor would reveal tissues, where silencing of transgene can not be taken over by the other systems.

The production of ta-siRNAs can be triggered by miRNAs thereby linking the two pathways (Allen et al., 2005; Axtell et al., 2006; Voinnet, 2005). Also, both miRNA and ta-siRNAs could, in addition to PTGS via mRNA degradation or translational inhibition, trigger further transcriptional silencing of the transgene by DNA methylation (Brodersen et al., 2008; Chellappan et al., 2010; Vaistij et al., 2002). Therefore, weaker effect of *nrpe1* on release of GFP compared to *ago1* and *dcl4* could also indicate that silencing of the GFP transgene by DNA methylation lies downstream of miRNA and ta-siRNA biogenesis pathways or act in a parallel manner (Figure 1.1, 1.5, 1.6).

It has been demonstrated that integration of a transgene can trigger production of siRNAs, which further lead to its silencing (Mourrain et al., 2007; Stam et al., 1998; Vaucheret et al., 2001). Although the endogenous *APUM9* has not been shown to trigger generation of siRNAs, it is then possible that integration of a second copy of the locus led to a switch from TGS to PTGS to silence the transgene (Nuthikattu et al., 2013).

Therefore, combinations of the above-mentioned pathways create several possibilities for further transcriptional (by DNA methylation) or post-transcriptional (by mRNA degradation or translational inhibition) silencing of the GFP transgene in silex. These possibilities can be investigated by closer studying the effect of

components of PTGS and additional components of TGS (such as DDM1, DRD1, MET1, CMT3) on expression of the endogenous *APUM9* and also on DNA methylation in the promoter of the GFP transgene as well as in the promoter of endogenous *APUM9.* Performing a genome-wide transcription profiling for the mutants of components of TGS and PTGS pathways in silex background would permit to detect possible changes in transcription levels of general TGS and PTGS targets, associated with presence of the GFP transgene. In addition, to check, whether the post-transcriptional silencing of the GFP transgene takes place via mRNA slicing or via translational inhibition, the effect of components of the PTGS pathway on mRNA level of *GAL4/VP16* and *GFP* should be tested. This can be achieved by performing a northern blot on total RNA from silex plants and mutants of components of the PTGS pathway, such as *ago1, dcl1, dcl4, ago4* and *rdr6.* Comparison of RNA blot results with the quantifications of protein levels of GAL4/VP16 and GFP in the same set of samples could provide an information on whether the PTGS of the GFP transgene acts through the mRNA degradation or by translational inhibition.

Role of SYD in silencing of the transgene in silex line

As a chromatin remodeler, SYD has been shown to control the transcription of flower homeotic genes. And, although an indication of it being involved in the suppression of the RdDM target *soloLTR* has been reported (Zhu et al., 2013), little is know about functions of SYD in gene silencing. However, participation of several other SWI/SNF ATPases in silencing have previously been reported. The known components of silencing machinery, such as DDM1, ATRX, DRD1, and MOM, although being structurally different from SYD, belong to the same SWI/SNF superfamily of chromatin remodeling ATPases (Brzeski, 2002; Chan et al., 2006; Jeddeloh et al., 1998; Higgs et al., 2000; Kanno et al., 2005; Richards et al., 1999; Verbsky and Richards, 2001; Plant Chromatin Database http:/www.chromdb.org; http://www.snf2.net). This suggests that SYD may play yet unknown general role in gene silencing.

This thesis reports for the first time that SYD can be recovered in a mutant screen designed to identify factors involved in gene silencing. The identified *syd-10* mutant is a weak allele of SYD. It displays common phenotypic characteristics with

the previously reported null-allele *syd-5* (SALK_023209, Alonso et al., 2003; Bezhani et al., 2007) during the vegetative stage, however *syd-10* displays weaker defects in flower development.

The delay in juvenile-to-adult phase change and decrease in total rosette leaf number observed for *syd* together with detected overaccumulation of anthocyanines in stems of *syd-10* plants, indicated that the miR156 pathway may be disturbed (Gou et al., 2011; Schwab et al., 2005; Todesco et al., 2010; Wu, 2006). Also, the prolonged delay in flower initiation of *35S::miR156/syd-10* plants indicated the additive effect of SYD and miR156 on regulation of flowering time.

Considering the obtained results, we assumed that the observed decrease in level of miR156 in stems and inflorescences of *syd* might be due to the fact that SYD is involved in a transcriptional regulation of miR156 precursors. Also, *SPL8* that is not targeted by miR156 was also affected in *35S::miR156/syd-10* plants as well as in inflorescences of *syd-5* and *syd-10*. This suggested a possible role of SYD in regulation of both, miR156-dependent and miR156-independent pathways of developmental timing.

Further, we demonstrated that SYD directly interacts with the promoter regions of some *MIR156* genes, affecting the transcription of miR156 precursors. The role of SYD in transcriptional regulation of miR156 has not been reported before. Also, we observed the enrichment for SYD in promoter regions of *SPL8* and *SPL3*. The synergistic functions of miR156-targeted SPLs and SPL8 in developmental phase change and reproductive tissue patterning were previously reported (Xing et al., 2010), supporting our proposition that SYD has a direct function in the regulation of developmental timing in *Arabidopsis*.

Down-regulation of miR156 level in silex by introduction of the miR156 mimicry target (*MIM156*) resulted in release of GFP expression in seedlings, indicating that miR156 (or a factor controlled by miR156) may take part in silencing the GFP transgene. However, it is not yet clear, whether miR156 targets the transgene directly, or its action is mediated through accumulation of secondary siRNAs (Figure 5.1). This would be interesting to examine in connection with the suggested above strategy for further characterization of silex line.

Considering the dual role of SYD in control of miRNA156 and its target *SPL3* (miR156-targeted transcription factor), SYD plays a role in spatiotemporal control of transcription of both miRNA and its target. Following the same principle, it is

possible, that in addition to transcriptional regulation of miR156 precursors, SYD may also affect the expression of another, yet unknown gene targeted by miR156. Further, this gene itself might be targeting the GFP transgene for transcriptional or post-transcriptional silencing.

Recently, the contribution of 21-nucleotide easiRNAs ("epigenetically activated") to targeting transposon transcripts has been reported (Creasey et al., 2014; Daxinger et al., 2009). This mechanism was suggested to primarily act in silencing of epigenetically reactivated TEs. Thus, as discussed in Chapter II, considering that the silex transgene contains a part of *ROMANIAT5* TE, it is possible that insertion of the GFP transgene in silex triggered the production of easiRNAs from the *ROMANIAT5*, which further lead to silencing to the GFP expression in some tissues. Also, the role of miR156 in easiRNAs production was suggested (Creasey et al., 2014), entailing that SYD might also be involved in transcriptional control of easiRNAs and its precursors. If this is the case, such connection could be revealed by testing the accumulation of known transposon-derrived easiRNAs in *syd* compared to the wild type plants (combined with earlier proposed experimental approaches).

However, as mentioned before, this action can still lead to either mRNA degradation or translational inhibition (Chellappan et al., 2010) (Figure 5.1). Also, siRNAs, targeting the transgene could initiate the establishment of DNA methylation, resulting in transcriptional repression through the RdDM pathway. Such combined action of the TGS and PTGS, involving different factors, would explain the complex control of the tissue specific silencing, that we observed (Figure 5.1).

Although we did not detect a direct interaction of SYD with the promoter region of the GFP transgene, it is still possible that SYD may target the transcriptional activation at a chromatin region next to the transgene, possibly the LTR of the *ROMANIAT5* TE (Figure 5.1). As the endogenous copy of this TE is not transcriptionally active, it would indicate the role of SYD in transcriptional activation of selected copies of the TEs, or its general role in suppression of TEs.

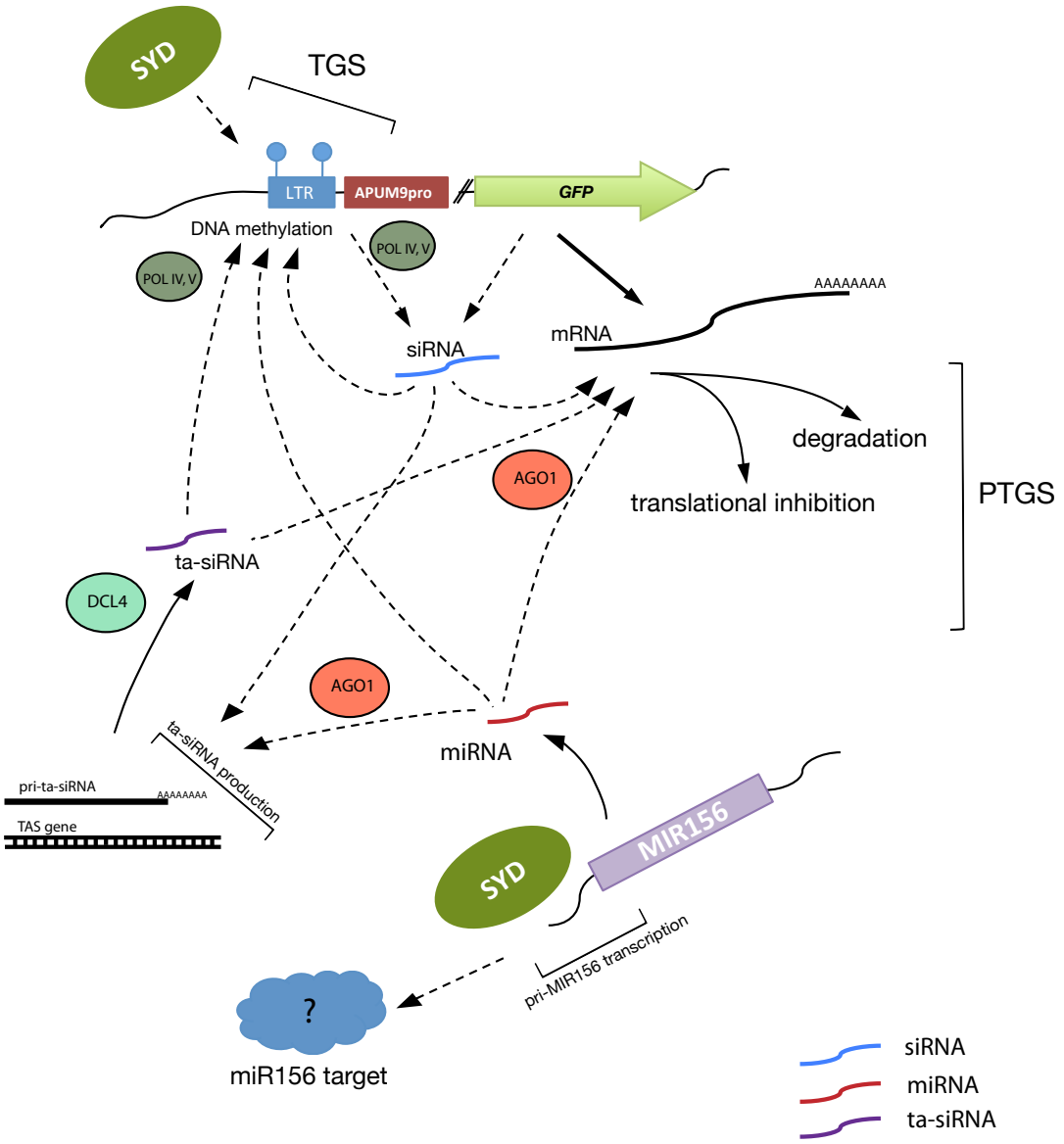


Figure 5.1 Model of interacting pathways of transcriptional and post-transcriptional gene silencing, regulating the expression of the silex GFP transgene

Model, schematically representing the mechanisms, possibly involved in silencing of the GFP transgene in silex. On transcriptional level, the transgene can be silenced by establishment of the DNA methylation in its promoter region. This can be a result of the Pol IV, Pol V dependent RdDM (As described in Chapter1), direct targeting by a miRNA, by tasiRNAs, derived from an unknown *TAS* gene, or as a result of silencing effect of chromatin remodeling by SYD. Also, several mechanisms might contribute to silencing the transgene at post-transcriptional level: targeting of the GAL4 or GFP mRNA by miR156-dependent ta-siRNAs can lead to mRNA degradation or translational inhibition. Bound by SYD miR156 may target the transcript of yet unknown gene, which itself could affect the silencing of silex transgene at transcriptional or post-transcriptional level.

However, since the GFP transgene contains the sequence of the endogenous LTR region of *ROMANIAT5* TE, the ChIP qPCR amplification of this region does not allow to reliably distinguish between the enrichment of SYD at endogenous and the transgene loci. A wider study of regions, bound by SYD on the whole genome level would provide information on whether it targets the regions of others silenced or active TEs.

Interestingly, it has recently been reported that the expression of at least two isoforms of miR156 (*MIR156A* and *MIR156C*) are negatively regulated by sucrose (Yang et al., 2013). At the same time, the first member of the SNF2 family was identified in *S. cerevisiae* as a regulator of sugar homeostasis (Neigeborn and Carlson, 1984). Yeast snf2p was shown to be required for transcriptional activation of *SUC2,* an invertase responsible for the anaerobic fermentation of sucrose (Abrams et al., 1986; Carlson and Botstein, 1982; Neumann and Lampen, 1967; 1969). Considering that both, miR156 and the SWI/SNF ATPases are highly conserved between the plant species (Axtell and Bowman, 2008; Cho et al., 2012; Cuperus et al., 2011; Flaus et al., 2006; Gong et al., 2013; Jerzmanowski, 2007), the functional connection between the miR156 and homologues of SYD may be present in other plants as well.

Figure 5.2 Difference in phenotypes of adult plants of *syd-10* and *syd-2*

The image shows adult plants of Col (WT), and 2 alleles of *syd* mutants from different accession backgrounds: *syd-10* (Col) and *syd-2 (Ler)*. The plants were grown at the same time in long day conditions.

However, the effects of such connection on plant development may differ between the species and even between accessions due to lower conservation of target sequences downstream of SYD and miR156. This can be illustrated by differences in phenotype of *syd* in *Columbia (Col)* and *Landsberg erecta (Ler)* backgrounds (Figure 5.2) in concert with the reported presence of the accession-specific markers at *LFY* and *AG* loci (Alonso-Blanco et al., 1998; Konieczny and Ausubel, 1993).

Our observation that SYD directly interacts with promoter regions of *MIR156* genes may indicate possible general function of SYD as transcriptional regulator of several others miRNAs. Supporting this suggestion, available ChIP-seq data for LFY, an interaction partner of SYD, indicate its occupancy in promoter regions of several *MIRNA* genes (Moyroud et al., 2011). In addition, SYD might also affect transcription of components of the sRNAs biogenesis pathways. This assumption can be supported by down-regulation of AGO7 mRNA level (a component of the ta-siRNA pathway, detected by the transcription profiling, data not shown, Hutvagner and Simard, 2008; Montgomery et al., 2008). However, to better understand functions of SYD in activation or suppression of genes, further investigation of SYD binding sites on whole genome level is required.

OUTLOOK

Epigenetic regulation of gene expression plays an important role in activation or suppression of genes, depending on cell type and the developmental stage. Currently, the precise epigenetic mechanisms, defining and directing the tissue specificity of gene expression are being actively studied in model organisms. In this thesis, we addressed the questions of regulation of tissue specific gene expression using the transgenic line with epigenetically controlled endogenous promoter. The results of our study revealed some aspects of the roles of TGS and PTGS in silencing of the transgene in silex transgenic line. However, several questions remain to be addressed to better understand the mechanisms, regulating the tissue specific silencing of GFP in silex.

Our current results indicate that release of GFP expression some tissues can be caused by factors of both, TGS and PTGS (lower part of inflorescence stems), whereas in the others only by components of PTGS (leaves, inflorescences). It is unclear, whether silencing of the transgene in some tissues is less stringent then in the others and if so, why. At the same time, it remains to be studied, what defines the specificity with which endogenous factors contribute to silencing of the GFP transgene silex line.

In this study, the connection between chromatin remodeler SYD, the highly conserved miR156 and the GFP transgene was demonstrated. However, It remains to be identified, whether miR156 directly targets the transgene, or acts via production of secondary siRNAs. Also, it is yet unknown, whether the silencing of the GFP transgene in silex by miR156 is achieved through establishment of the DNA methylation, mRNA degradation, or by translation inhibition. For better understanding if the silencing mechanism, involved in repression of the transgene in silex, all these possibilities need to be further investigated.

Also, the results of this study indicated possible general the role of SYD in silencing. Further genome-wide studies of SYD binding regions are required to study new targets of SYD. This study should also be combined with investigation of the mechanisms involved in target recognition and cooperation with other components of chromatin remodeling complex.

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Appendix I

Primer name	Nucleotide sequence
Chapter II	
T-DNA ins F	GGTGTGGAAACCGGCGGGAG
T-DNA ins R	CCAACTAGCCACGAGCTCCCCT
ROM_LTR bis F2	AAGGGGGAGTGTTATAAAYTTGATAAGTATGG
ROM_LTR bis R2	CTTCATTRTTRRATCATCAACCACTTT
Apum9 pro_R	ATCCATCACTCATCTCTATCCATAA
LBb1.3	ATTTTGCCGATTTCGGAAC
nrpe1-2 LP	GCTTTGACCCGATCCTTAAAC
nrpe1-2 RP	GGGTTCCAGGGACAAAAATAA
Ago1-F	TTAGGCCCGCGTGTGCTTCT
Ago1_R	GGGCACTTCTCGACCTGCTCAT
se-1 F	TGGCGTGTTCATGGTCTGGACT
se-1 R	TGCAGCCCTTGGCTCCACAA
sgs3-13 LP	AAGGCCATGCTTGTACATGAG
sgs3-13 RP	TATGAGGCTCTTAGAGCACGC
SAIL LB1	GCCTTTTCAGAAATGGATAAATAGCCTTGCTTCC
dcl4-2LP	TTTGCCAGTCTTACAAGTGGG
dcl4-2RP	GAGGCACCATATAGCAGCTTG
Chapter III	
GFP_QTF	TGGCCGACAAGCAAAAGAAC
GFP_QTR	CCGCCGTCTTCGATGTTG
ACT2_QTF	TGCCAATCTACGAGGGTTTC
ACT2_QTR	TTACAATTTCCCGCTCTGCT
UBQ_QTF	GGCCTTGTATAATCCCTGATGAATAAG
UBQ_QTR	AAAGAGATAACAGGAACGGAAACATAG
SYDm F	CGACCATTTGTTCTGCGTCGGC
SYDm_R2	AGAGCACCACGATCACCCCC
SYDm_R	TGCGCTAACCAGTGCTTCATTGC
SYDseq1_F	TCGCAGAGACGACGCATAGC
SYDseq1a_F	TGTTTTCCTTATATATCGTCGCTGGT
SYDseq1_R	AGGCACTGGGCTCTGAGCTG
SYDseq2_F	AGAGGGATTCCTGCCTTCAGGT
SYDseq2a_F	GCAACCGTCGCCTAAGTACACC
SYDseq2_R	TCTCACGGTGAAGTCGTTCCT
SYDseq3_F	AGGAGAGAAAGGTTGAAGGGTTTC
SYDseq3a_F	CCATCGTCCCTAGTGGGTGGA
SYDseq4_F	GCCAACTGCATTCAGAGGAGGT
SYDseq4a_F	TTTCCACAATGACAAGGCTTCTT
SYDseq4_R	TCTTTCCTTTGTTTGTCGATGGACTC
SYDseq5_F	CTTTCTTGCAGATCTGACATTTTTGT
SYDseq5a_F	AAGCCTTGACTCCGGTATCATT
SYDseq5_R	TCATGACTCGAGCAACATCAGT

SYDseq6_F	ACCTCTGGTACTGGTGGTTCTGC
SYDseq6a_F	AGAGAAGCTATCCTCCTAAGTTCG
SYDseq6_R	TGTTCAGGGTCTATCCCACCA
SYDseq7_F	ATCTGTGCAGGTCCCGGATGC
SYDseq7a_F	CCAGGCCGAGCCATCCAATTT
SYDseq7_R	TGTTTCCTCTAAGTGCATGTGTGTACC
SYDseq7a_R	TGAAAGAGTGATCAGAAATGATTCTAAGCC
pSYD CAMBIA1304 - SYDproF	AAGCTTGGTTCATGGGAACCAAGGAGAAAAGATTATTGG
pSYD CAMBIA1304 - SYDproR	CTTAACTTAAATTACACCATCACATAAGAATTCCCTGCCC ATGGT
SPL3_QTF	ACGCTTAGCTGGACACAACGAGAGAAG
SPL3_QTR	TGGAGAAACAGACAGAGACACAGAGGA
SPL9_QTR	AGTCTCCTGCGGCAACTCCTTT
SPL9_QTF	TCGAGACACCGAGTTTGTGGAGTG
Rprimer Syd probe	TAATACGACTCACTATAGGGACTCCAGTCCACCCCACCAT CT
miR156A_chipQTF	CAAAGAGAGGGAGGGAGAGAGGGA
miR156A_chipQTR	CCCTAGATTTGATCTTCTAAAGGGTCTCAAATGG
miR156C_chipQTF	TCCCTCTTACATGCAATGGGACAGATG
miR156C_chipQTR	AGGCTTGTCGTTGCCGTTTATAGGT
miR156H_chipQTF	AGAGATGTGACGTGCGGCGT
miR156H_chipQTR	GCCGGTCTCCACCTCTTATTCACC
EIF4A1_chipQTF	CTTTTTCGGATTTCCGGTTTTACCCCTT
EIF4A1_chipQTR	TGTTGGAAAACCTTGTCCGAGCCA
SydProbe_R_XhoI	GTCTCGAGACTCCAGTCCACCCCACCATCT
SydProbe_F_BamHi	CATGGATCCCTGGTAGGCAGCTTGGTGGATCA
Chapter IV	
qMIR156A_F	TGCACTTGCTTCTCTTGCGTGC
qMIR156A_R	ACAGGCCAAAGAGATCAGCACCG
qMIR156C_F	AAGAGAAACGCATAGAAACTGACAG
qMIR156C_R	GGGACCGAATCGGAGCCGGAATCTGAC
qMIR156H-F	GAAAGAGAGCACAACCTGGGATTAGC
qMIR156H-R	CGCAATGATGGTGGCAGAAGGAAAGAG
SPL3_chipQTF	AACGCAATGTGGTATGTTTGGTCTGTT
SPL3_chipQTR	GTGCTAATGTAGTGATTGGAAAAGCAAGACTG
SPL8_QT F	CGACGACTTCGTGAGCAGGCTA
SPL8_QT R	TGGCTCAGATCCGCGTTGCAT
SPL8_chipQT	ACCGACATGTCTCCTCCCCCTT
SPL8_chipQT	GCAATCCCGAGGAAGATCTCTCTCTCTTT
SPL11_QTF	GTCCAAGTTTCAACTTCATGGCG
SPL11_QTR	GAACAGAGTAGAGAAAATGGCTGCAC
SPL11_chiPQTF	TAGCTTGCGAGGGAGGGACCTT
SPL11_chipQTR	AACGGCAGCAAGCTCAGCCA

List of Abbreviations

aa	amino acid
AG	AGAMOUS
AGO	ARGONAUTE
AP	APETALA
ARF	AUXIN RESPONSE FACTOR
bp	base-pair
C	carbon
CaMV	cauliflower mosaic virus
cDNA	complementary DNA
Col-0	*Arabidopis thaliana* ecotype Columbia
DCL	DICER-LIKE
DNA	desoxyribonucleic acid
dNTP	deoxy-nucleoside-5'-triphosphate
ds	double strand
EDTA	ethylenediamine tetraacetic acid
et al.	*et alii* (Latin = and others)
etc.	*et cetera* (Latin = and so on)
GFP	green fluorescent protein
GUS	β-glucuronidase
h	hours
H	hydrogen
HDACs	histone deacetylases
HEN	HUA ENHANCER
HST	HASTY
HYL	HYPONASTIC LEAVES
kb	kilo base-pairs
kDa	kilodalton
LD	long day
M	Mole
mM	millimolar (10^{-3} mol/dm^3)
µM	micromolar (10^{-6} mol/dm^3)
min	minute
ml	milliliter (10^{-3} Litre)
µl	microlitre (10^{-6} L)
µm	micrometer (10^{-6} m)
NASC	Nottingham Arabidopsis Stock Center
OD	optical density
PCR	polymerase chain reaction
PHB	PHABULOSA
PHV	PHAVULOTA
pre	precursor
pri	primary
Pol	polymerase
PTGS	post-transcriptional gene silencing
RdDM	RNA-directed DNA methylation
RISC	RNA-induced silencing complex
RNA	ribonucleic acid
RNAi	RNA interference

RT	room temperature
SD	short day
SDS	sodium dodecyl sulfate
SPL	SQUAMOSA PROMOTER BINDING PROTEIN LIKE
ss	single-stranded
ta	trans acting
TGS	transcriptional gene silencing
Tris	tris(hydroxymethyl)-amino-methane
TrisHCl	tris(hydroxymethyl)-amino-methane hydrochloric acid
Tween20	polyoxyethylene-sorbitan monolaurate
UV	ultraviolet
WT	wild-type